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Use of an *ex vivo* model of human colorectal tumours to study response to the MEK1/2 inhibitor AZD6244

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Declaration

I declare that the research described within this thesis is my own work and that this thesis was composed by myself unless otherwise stated.

Neither this thesis nor any part thereof has been submitted for any other degree or professional qualification.

Name:

Signature:

Date:

Abstract

Colorectal cancer is the second most common cause of cancer death in Western Europe and North America. Current therapies are largely ineffective and are associated with considerable morbidity. Activating mutations in *KRAS* and *BRAF* genes are frequent in colorectal cancer, especially at later stages of the disease, and result in constitutive activity of the MAPK pathway, leading to increased proliferation and tumour survival. The MEK1/2 inhibitor AZD6244, that targets the MAPK pathway downstream of these mutations, has been tested as novel therapy for colorectal cancer. However, clinical trials have been disappointing due to an apparent intrinsic and/or acquired resistance to treatment. Mechanisms underlying this resistance have been studied using cell lines and tumour xenografts. However, the relevance of these data to advanced human colorectal cancer is unclear.

One of the difficulties in testing and developing novel therapies for colorectal cancer is the lack of representative models of human disease. Thus, the initial aim of my PhD was to develop a method to culture human colorectal cancers *ex vivo* in order to use this as a platform for investigating response to AZD6244 and other therapies. These studies indicated that regardless of growth conditions, colonic tumour explants suffered extensive apoptosis in the first 24h in culture, which limited their application in drug response assays. Therefore, as an alternative to long term culture of human colorectal explants, I tested the effects of AZD6244 using acute treatments. Twenty three fresh colonic tumours were obtained from patients and treated for 1h with AZD6244 *ex vivo* in dose response studies. In all samples, MEK1/2 inhibition occurred within 1h of treatment. In one group of particularly sensitive tumours, the drug also had a distinct phenotypic effect. In these tumours, I

found that the agent induced a dose-dependent decrease in proliferation and increase in apoptosis within 1h of treatment. Analysis of markers for this sensitivity indicated it was not clearly dependent of the presence of *KRAS* or *BRAF* mutations, which have previously been shown to confer sensitivity. Other markers of sensitivity / resistance were also examined. In addition to studies with AZD6244 alone, I examined the combined effects of this agent and aspirin in colon cancer cells lines and in tumour explants, with promising results. Whilst the use of fresh patient tumour tissue has some technical and logistical challenges, these data suggest that such methodologies are worthy of further investigation as a means to examine determinants of sensitivity and resistance to novel therapies, or their likely activity in combination.

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Table of contents

Table of figures

Chapter 1: Introduction

1.1 - Colorectal Cancer

Colorectal cancer is the third most commonly diagnosed type of cancer worldwide (*1*) with an incidence of over 1 million reported every year (*2*). It is the third most commonly diagnosed cancer in males and the second in females (*3*), with approximately 80% of patients being diagnosed between the ages of 55 and 85 years old (*4*). Despite advances in surgical techniques and adjunctive therapies the overall 5-year survival rate is still approximately 50% (*5*).

Colorectal cancers develop in a stepwise fashion from normal epithelial mucosa to benign tumours or adenomas, and into malignant tumours or carcinomas as a result of two major processes: microsatellite instability caused by defective DNA mismatch repair or chromosomal instability. The former accounts for approximately 15% of colorectal cancers and the latter is responsible for 85% of cases (*6*). Carcinomas have the ability to invade other tissues underlying the intestinal epithelium and as a result tumours eventually acquire the capacity to migrate to other organs and form metastasis. While the progression from adenomas to carcinomas occurs through a period of many years, the development of metastatic disease is a much faster process (less than 2 years), and few events are needed for a cell population with high invasive potential to acquire the ability to metastasize (*7*).

Colorectal cancers are routinely classified according to Dukes and TNM systems both of which determine the local extent of the disease. Dukes classification, which is based on histopathological information obtained from microscopic examination of resected tumours, originally separated tumours into 3 categories: an A tumour remained confined to the intestinal wall, a B tumour had spread beyond the intestinal wall but had not invaded the lymph nodes, and C tumours had already

formed lymph node metastasis. A D category was later on added to indicate the presence of distant metastasis (*8*).

The TNM (Tumour, Nodes, Metastasis) system includes clinical and pathological staging approaches, and as it can be applied to pre-operative evaluation of patients provides a more comprehensive classification than the Dukes system. The TNM system is also constantly updated to reflect the most recent and robust data. In this system, T refers to the extent of the primary tumour at the time of diagnosis, N refers to the lymph node status, and M refers to the presence of distant metastatic disease. A "p" or "c" used as a prescript designates the pathological or clinical determination of the TNM stage, respectively. Tumour stage is usually referred to as number I, II, III or IV which represents the TNM information grouped by prognosis. A higher number indicates a more aggressive tumour and a worse outcome (*9, 10*).

Dukes and TNM staging systems are useful to stratify patients, define therapeutic approach and determine prognosis based on tumour morphology and the local extent of the disease. However, it is not uncommon that patients with tumours with similar characteristics present very different outcomes. Also, some tumours present atypical morphologies or lack characteristics essential for classification and cannot therefore be placed into a definite category which might result in inaccurate diagnosis and consequent inadequate patient care (*11*). Molecular characterisation and staging of tumours based on molecular markers and gene expression signatures, together with histopathological characterisation would provide better insight of the tumour's individual properties, and a fundamental tool to define adequate therapeutic strategies and accurately predict long term prognosis (*12, 13*).

Despite the use of comprehensive classification systems and recent advances in anti-cancer therapies, the outcome of patients with advanced colorectal cancers is still very poor. While 90% of those diagnosed with localised tumours survive after 5 years, the survival rate drops to 67% for those diagnosed with lymph node disease, and is dramatically decreased to less then 10% for patients with metastatic tumours (*14, 15*). Currently available colorectal cancer therapies are still largely ineffective against the later stages of the disease with metastatic cancers presenting the biggest challenge in terms of therapeutic intervention.

1.2 – Current Therapies

The complex network of events that define colorectal cancer and other cancers makes it difficult to design therapies that efficiently eradicate tumours. Nevertheless, chemotherapies aimed at invasive colorectal cancers have greatly evolved in the past 50 years since the discovery of 5-fluorouracil, the development of the cytotoxic agents, oxaliplatin, irinotecan, and capecitabine, and the use of combined chemotherapies (*16*). Recently, targeted therapies using monoclonal antibodies such as Bevacizumab (Avastin), Cetuximab (Erbitux) and Panitumumab (Vectibix) have been developed.

Bevacizumab is a humanised monoclonal antibody against vascular endothelium growth factor (VEGF), an important pro-angiogenic protein overexpressed in tumour cells (*17, 18*). Inhibiting VEGF results in blood vessel regression, and inhibition of formation of new vessels with consequent reduction of blood supply and tumour growth (*19*). Cetuximab is a chimeric IgG1 monoclonal antibody and Panitumumab is a fully humanised monoclonal antibody. Both

therapies target the epidermal growth factor receptor (EGFR) which is involved in cell differentiation, proliferation and survival, migration, angiogenesis and apoptosis (*17, 20, 21*).

The use of targeted therapies, alone or combined with cytotoxic agents, is still somewhat controversial. While some authors claim that it can significantly increase the life expectancy of metastatic colorectal cancer patients (*19, 22*), others suggest that treatment with these therapeutic options does not improve progression free survival, overall survival and quality of life (*21, 23*). Also, the outcome of these therapies is variable as not all patients respond to treatment, and the mechanisms that affect their efficacy are still not fully understood.

To date no proven biomarkers of efficacy of anti-VEGF therapy have been identified. The presence of single nucleotide polymorphisms has been suggested as patients carrying particular SNP's presented better overall survival, though the mechanisms through which the presence of specific SNP's increases response to treatment are still unclear (*23*). The presence of VEGF isoforms has also been suggested as a marker of response to anti-VEGF targeted therapies. Alternative RNA splicing originates four VEGF isoforms with specific affinities to the extracellular matrix. The smaller VEGF isoform, which lacks the ability to bind to the extracellular matrix, circulates in the plasma and provides a more accurate indication of the VEGF levels produced by a tumour. Metastatic breast cancer patients with high levels of this isoform presented better progression free survival and overall survival after treatment with anti-EGFR therapies. However, no such correlation was observed in metastatic colorectal cancer patients (*24*).

The mutational status of *KRAS* is a good indicator of the outcome of anti-EGFR therapies as patients with mutant KRAS tumours rarely respond to treatment with Cetuximab (*25*). However, anti-EGFR therapies are also only effective in 60-70% of patients with wild type *KRAS* tumours (*26*). In these tumours, *BRAF* mutational status, EGFR amplification and PTEN expression have been proposed as indicators of response to treatment with Cetuximab (*25, 27*). The use of targeted therapies is also limited by serious side effects (*28*).

Despite advances in cancer therapy, patients diagnosed with metastatic colorectal cancer still face a very poor prognosis. Given the now known association between a tumour's individual characteristics and response to treatment, it is fundamental to understand the mechanisms of action of available therapies in order to identify patients more likely to benefit from them. There is also a pressing need for the development of new therapeutic options for those patients that do not respond or present severe side effects to the currently available ones. These new therapies should target the pathways involved in the initiation and progression of colorectal cancer so that a wider and more effective range of therapeutic solutions can be offered to all patients. In order to achieve these aims, a study model that better represents the complexity of human disease and the individual characteristics of each tumour is needed.

1.3– Initiation of colorectal cancer

In recent years, the idea that colorectal cancers arise and are maintained by a small number of cells with stem cell like properties such as self-renewal and pluripotency has been suggested. Cancer stem cells are thought to derive from normal intestinal stem cells with deregulated functions that eventually lead to neoplastic transformation (*29*). This notion is supported by the fact that β-catenin, which is strongly involved in the development and progression of colorectal cancer, also plays a fundamental role in the regulation of the intestinal stem cell niche (*30*).

The human intestinal epithelium is a rapidly proliferating tissue that self renews every five days. In the normal epithelium a tight balance is constantly maintained between proliferation, migration and differentiation in order to assure the tissue's continuous renewal (*31*). Figure 1.1 shows a schematic representation of the intestinal crypt and the mechanism of proliferation and differentiation of the different cell types that form the intestinal epithelium, as they progress upwards in the crypt.

In the normal intestinal epithelium there are two main compartments where stem cells have been shown to be located: the bottom of the intestinal crypts, and the +4 position immediately above the Paneth cells. The location of the stem cell compartment at +4 position was proposed in the 1950's and later on demonstrated by Potten *et al* using label retention assays (*32, 33*). Barker *et al* investigated the role of a group of cells located at the base of the intestinal crypts known as crypt base columnar cells that express Lgr5 (Leucine-rich-repeat containing G-protein-coupled receptor 5).

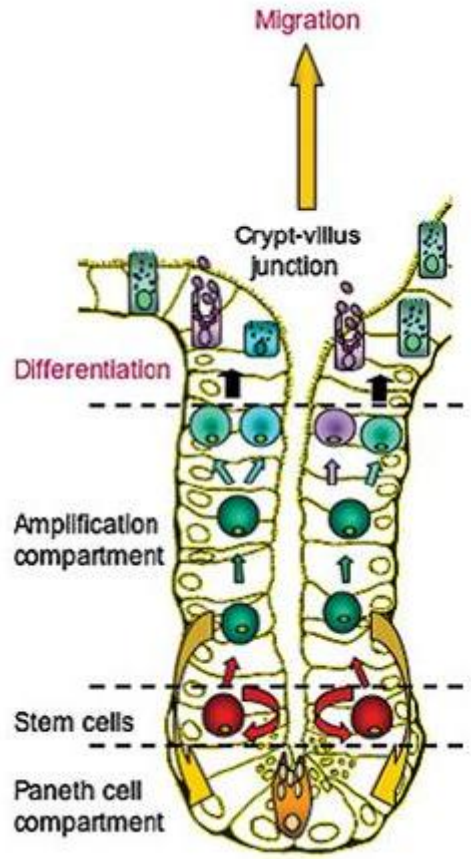


Figure 1.1: Schematic representation of the intestinal crypt. Stem cells divide and start migrating upwards in the crypt towards the villus in the small intestine or the lumen in the colon, as they differentiate into the different types of cells that form the intestinal epithelium. Paneth cells in the small intestine and Goblet cells in the colon migrate to the bottom of the crypts where they form a niche with Lgr5 (+) stem cells. Adapted from Pinto *et al* (*31*))

This protein was identified as a Wnt target and is expressed in the crypts but not villi of mouse intestines. Using lineage tracing experiments, the group demonstrated that all 4 types of differentiated intestinal cells originated from these particular group of Lgr5 expressing cells (*34*). These findings were shortly after supported by observations that single sorted Lgr5(+) stem cells have the ability to originate crypt-villus structures in culture (*35*). There is therefore strong evidence that both the cells located at the +4 position, and the Lgr5(+) cells located in the crypt base are stem cells capable of originating all intestinal epithelial lineages. However, the nature of the relationship between the two stem cell compartments has, until recently, been poorly understood. In a recent publication, Tian *et al* (*36*) demonstrated that, upon tissue injury, slow cycling label retaining Bmi1(+) cells located at position +4 are capable of originating Lgr5(+) cells and subsequently repopulating the intestine. This suggests that the rapidly cycling Lgr5(+) cells are not fundamental for intestinal homeostasis, and that in their absence Bmi1(+) cells provide an alternative stem cell pool. Interestingly, this work was shortly followed by the work of Takeda *et al* (*37*) who demonstrated that each stem cell pool is capable of giving rise to the other suggesting a bidirectional lineage relationship between the two stem cell compartments. Though the work present by these authors provides an explanation for the presence of two stem cell compartments in the intestine, more work is needed to fully elucidate the mechanisms that regulate homeostasis between these two groups of stem cells.

The concept of cancer stem cell dates from over 30 years ago and it originated from observations that only a small group of cancer derived human

myeloma cells were capable of originating colonies when cultured in soft agar (*38*). The cancer stem cell hypothesis proposes that cancer stem cells originate from normal stem cells with deregulated cellular functions that lead to neoplastic transformation. This notion is supported by the fact that features present in normal stem cells like the capacity to indefinitely self renew and to differentiate into various cell types as well as high telomerase activity are also found in cancer stem cells (*29*). It is therefore legitimate to assume that markers used for the identification of normal epithelial stem cells would also be present in cancer stem cells originating from the same tissue. The expression of Lgr5 was therefore used by Barker *et al* to demonstrate that the proposed intestinal stem cells can also originate and maintain adenomas in mice upon loss of APC (*39*). A similar study was performed by Zhu *et al* who demonstrated that a specific group of cells located at the base of the intestinal crypts and co-expressing Lgr5 and Prominin1 (Prom1 or CD133) were responsible for the maintenance of the whole intestinal cell lineage, and were also capable of originating adenomas upon activation of Wnt signalling in the presence of mutant β-catenin (*40*). The use of CD133 as a marker for colorectal cancer initiating cells was also demonstrated in mouse xenograft models (*41, 42*), though other authors have also presented evidence that CD133 expression is not restricted to intestinal stem cells or cancer initiating cells, and that this protein has no obvious functional role in tumour development (*43, 44*). Other markers or combination of markers such as EpCam, CD44, CD166, CD29, CD24, and Bmi1 have also been proposed (*45-48*) but the significance of these markers still generates some controversy.

Though the theory that cancer stem cells are the driving force behind the initiation and maintenance of colorectal cancer is well established in murine models

of intestinal neoplasias, this has not yet been confirmed in human tumours. Nevertheless, colorectal cancer stem cells have recently been proposed as promising targets for therapeutic intervention (*49*). As the cancer stem cell field is rapidly evolving, and tools for the identification of markers become apparent it will soon be established if this can be applied to the human disease.

Regardless of whether it occurs in stem cells or in any other cells that form the intestinal epithelium, colorectal cancer initiation and progression results from two main events: activation of oncogenes and the mutational inactivation of tumour suppressor genes. A total of four or five mutations in these genes are needed for the formation of a malignant tumour, but fewer mutations are necessary for the development of the benign form of the disease. Fewer or no additional mutations are also thought to be needed for carcinomas to progress towards metastatic disease (*7, 50, 51*).

Colorectal cancer initiating mutations can occur sporadically or due to inherited predisposition. Sporadic colorectal cancers account for 75-80% of cases (*52*) while 3% of tumours are found in patients with Lynch Syndrome or Hereditary Non Polyposis Colorectal Cancer (HNPCC) (*53*), and 1% occur in patients with Familial Adenatous Polyposis (FAP) (*54*). The causes leading to the development of the remaining 15% of colorectal cancers are still unclear (*55*). FAP is caused by the mutation of the tumour suppressor gene Adenomatous Polyposis Coli (*Apc*) while HNPCC originates from mutations in mismatch repair genes such as *MSH2*, *MLH1*, *PMS2* and *MSH6* (*56, 57*).

Somatic *Apc* mutations are also present in approximately 70% of sporadic colorectal cancers and are considered an initiating event in the development of the disease (*58*). The APC protein is part of the complex responsible for the degradation of β-catenin in the cytoplasm. Some tumourigenesis models suggest that when APC is mutated, β-catenin is translocated to the nucleus where it activates the transcription of several genes involved in carcinogenesis (*59*). However, other reports indicate that APC mutations alone are not sufficient to induce nuclear accumulation of β-catenin (*60, 61*). Mutations of the *β-catenin* gene are also present in the early stages of tumourigenesis and are found in approximately 50% of sporadic colorectal tumours carrying wild type APC. Mutations in APC and β-catenin are mutually exclusive (*31, 62, 63*).

1.4 – Progression of colorectal cancer

Colorectal cancer progression from adenomas or adenomatous polyps, into carcinoma, and into metastatic disease occurs through continuous accumulation of mutations in genes like growth factors and respective receptor genes, cell cycle checkpoint genes, and apoptosis related genes. These mutations follow loss of APC and are acquired at different stages of cancer development (*56*). As progressively more aggressive tumours continue to accumulate mutations, it is the number and not the sequence of these mutations that defines the tumour's individual biological properties (*50*). These individual properties will later on define patient response to treatment.

Mutations in the tumour suppressor gene *p53* are one the most common events in the development of colorectal cancer playing a critical role in

carcinogenesis and tumour progression as this protein is involved in the regulation of cell cycle, apoptosis and DNA repair (*64*). Mutations in this gene have been found in over 40% of colorectal tumours and are associated with poor prognosis (*65*).

Another common event in the development of colorectal cancers is allelic losses on chromosome 18 (*66*). This region encodes for the tumour suppressor genes *Smad2*, *Smad3* and *Smad4* which mediate TGF-β signalling, and therefore the presence of mutations in these genes is also associated with colorectal cancer progression (*67*). Figure 1.2 shows a diagram of the genes most commonly involved in the initiation and progression of colorectal cancer.

Mutations in KRAS, a member of a family of proteins responsible for the regulation of signal transduction pathways involved in cell proliferation, differentiation, migration and apoptosis, are thought to follow APC mutations, promoting the progression from adenomas into carcinomas (*68, 69*), and are now known to be required for nuclear translocation of β-catenin together with homozygotic *Apc* mutations (*70*). KRAS is involved in the activation of several pathways, of which the best characterised is the ERK1/2 pathway, and mutations that lead to up-regulation of KRAS are fundamental for the maintenance and progression of colorectal cancers (*71*). A much higher frequency of *KRAS* mutations has been found in sporadic colorectal adenomas when compared to adenomas from patients with FAP which suggests that different molecular mechanisms are involved in the process of carcinogenesis between these two types of the disease (*72, 73*).

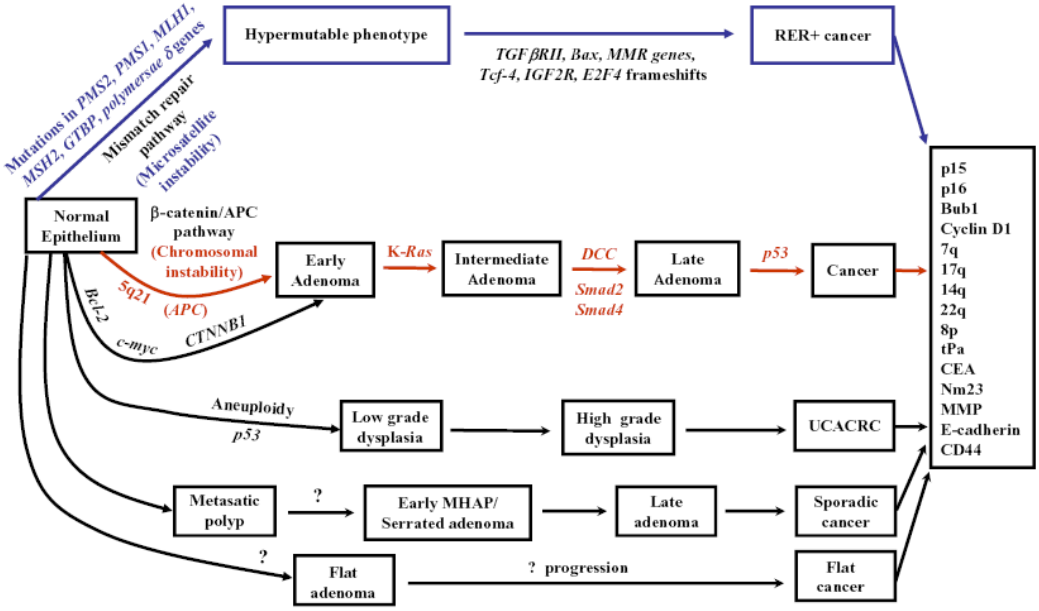


Figure 1.2: Model for genetic alterations in the development of colorectal cancer. Two main pathways involved in the development of colorectal cancer: mutations in the APC gene followed by mutations in KRAS and p53 (red arrows); and mutations in mismatch repair genes and microsatellite instability (blue arrows). Black arrows show less characterised pathways that are nevertheless related with the two main ones. (Adapted from Narayan *et al* 2003 (*56*))

1.4.1 – The Ras/Raf/MEK/ERK pathway

The mammalian MAP Kinase pathways which include four distinctly regulated groups: the extracellular signal regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38 and ERK5 pathways, are signalling cascades that allow communication between the cell membrane and the nucleus. These pathways play an important role in controlling cellular physiology and are often deregulated in disease states (*74, 75*).

The ERK1/2 pathway is involved in a variety of cellular processes such as cell cycle regulation, proliferation, survival, differentiation and apoptosis all of which are fundamental for normal development (*76, 77*). In normal cells, the ERK1/2 pathway is activated in response to extracellular and intracellular stimuli such as growth factors, serum, phorbol esters, cytokines, osmotic stress and microtubule disorganisation (*78*). The ERK1/2 signalling cascade is initiated by activation of cell surface receptors and regulated by the binding of a Ras GTPase family member (Hras, Kras or Nras) to a member of the raf kinase family (C-raf, A-raf or B-raf). Raf family members then phosphorylate MEK1/2 which in turn activate ERK1/2 (*79, 80*). A schematic representation of the pathway is shown in Figure 1.3.

ERK1/2 has a variety of targets that range from other kinases, phosphatases and transcription factors which explains why this pathway is involved in such diverse cellular functions. Factors such as cell type and cellular localisation determine which targets are activated and when (*81*).

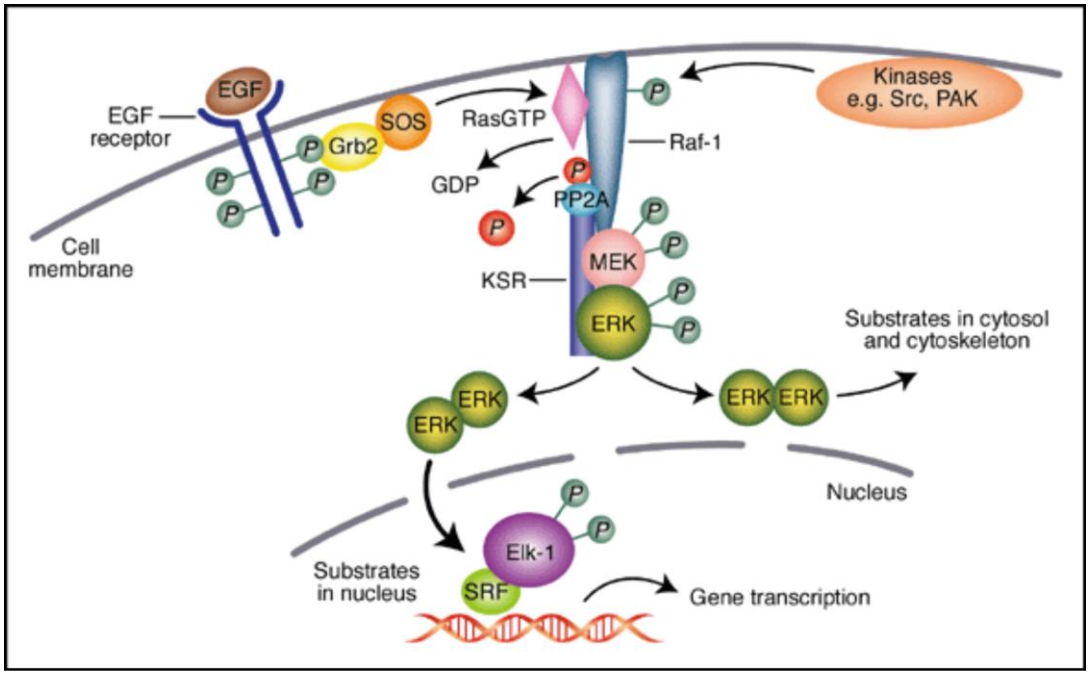


Figure 1.3: Schematic representation of the Ras/Raf/MEK/ERK pathway.
(Adapted from Kolch *et al* (*81*))

Upon activation of the pathway, a great proportion of ERK1/2 is quickly translocated into the nucleus where it activates several substrates such as SRC-1, Pax6, NFAT, Elk-1, and MEF-2, c-fos, cjun, c-myc and STAT (*78, 80*). ERK1/2 remains in the nucleus during G1 phase of the cell cycle, playing a fundamental role in the progression to S phase (*82*). However, ERK1/2 alone is not sufficient to induce cell cycle progression (*83*), and once in the nucleus activates Elk-1, Sap-1a and TIF1A which are also involved in the mitotic process and cell growth (*84-86*). ERK1/2 is then rapidly inactivated at the G1-S phase transition and exported from the nucleus (*87*). ERK1/2 can also promote cell survival by affecting FOXO transcription factors and perturbing activation of genes responsible for the induction of apoptosis like *Bim* and *FasL*, and genes involved in cell cycle regulation like *p27* and *Cyclin D*, or by destabilisation of both Bim and Bad (*88*). ERK1/2 is also known to activate cytosolic and membrane proteins PLA2, CD120a, Syk and calnexin; and cytoskeleton proteins like neurofilaments and paxillin (*78, 80*).

Though activation of the ERK1/2 pathway is generally associated with proliferation and survival, there is also evidence that, depending on cell type and stimuli, ERK1/2 can also induce cell death. Retention in the cytoplasm seems to be an important factor for ERK1/2 induced apoptosis (*88*). Park *et al* have shown that sustained ERK1/2 activation can induce cell cycle arrest in various cell types (*89*), and reports of ERK1/2 induced apoptosis in response to DNA damage can also be found in the literature (*90, 91*).

The Ras/Raf/MEK/ERK pathway is tightly regulated by a series of negative feedback mechanisms that control the magnitude and duration of the signal and determine which cellular functions are promoted. These mechanisms can be short

term ERK1/2 negative feedback phosphorylation of upstream members of the pathway or long term effects that require new protein synthesis. Phosphorylation of the Ras exchange factor SOS by ERK1/2 induces disassembly with Ras and termination of its activation (*81, 92*), Raf phosphorylation by ERK1/2 inhibits Raf's ability to phosphorylate MEK1/2, and phosphorylation of Receptor Tyrosine Kinases (RTK) by ERK1/2 inhibits signal output upstream of Ras. Long term effects involve ERK1/2-dependent induction of transcription of Dual Specificity Phosphatases (DUSPs) which can de-phosphorylate and inactivate ERK1/2 (*92, 93*).

Deregulated ERK1/2 pathway is present in about one third of all cancers (*78*). Up-regulation of the ERK1/2 pathway results from constitutive activation of cell surface receptors or gain of function mutations in ras or raf family members (*94, 95*) leading to amplification of signals involved in a variety of cellular functions that sustain tumour proliferation and survival (*96*). Mutations in the *ras* gene are present in approximately 30% of all cancers (*68*) and the frequency of mutations of each ras family member varies with cancer type (*97*). In colorectal cancer, the most frequently mutated member of the ras family is KRAS with a frequency of over 40% (*98, 99*). *BRAF* mutations occur in 12% of colorectal cancers (*98*). As *KRAS* and *BRAF* mutations are mutually exclusive their combined frequency amounts to over 50% of all colorectal cancers (*100*).

There is substantial evidence that *KRAS* and *BRAF* mutations contribute to the maintenance and progression of colorectal cancers. Disruption of the single mutant *KRAS* allele induced loss of tumourigenic ability of colorectal cancer cell lines implanted in immnuno-compromised mice (*101, 102*). Recent reports also show that mutant KRAS and its effector RAF1 are necessary for tumour invasion and

formation of metastasis (*103*). Matos *et al* have shown that mutant BRAF functionally cooperates with Rac1b to sustain colorectal cancer cell viability, providing an alternative survival pathway in cells carrying wild type KRAS (*104*).

The presence of *KRAS* and *BRAF* mutations has been associated with poor prognosis and resistance to treatment with anti-EGFR therapies in colorectal cancer patients (*105, 106*). Targeting the pathway downstream of these mutations is therefore a promising therapeutic option.

1.5 – MEK1/2 inhibitors

To date, several MEK1/2 inhibitors have been developed but only a few have reached clinical evaluation. The first MEK1/2 inhibitor to be developed was PD98059 which inhibited both inactive forms of MEK1 and MEK2 but failed to act on the phosphorylated form of the proteins (*107*). Two other MEK1/2 inhibitors were identified, U0126 and Ro 09-2210, but due to their pharmaceutical limitations none of these compounds were even taken into clinical evaluation. Nevertheless, PD98059 and U0126 provided useful tools for the study of ERK1/2 pathway functions in normal and malignant cells (*108*).

Pfizer's CI-1040 was the first MEK1/2 inhibitor to enter clinical trials. This compound is a potent non-ATP and non-ERK1/2 competitive MEK1/2 inhibitor that was shown to inhibit the growth of colon tumours by a maximum of 80% in mouse and human xenografts models (*109*). However, the use of this compound in the clinic was limited by insufficient anti-tumour activity, poor solubility and low bio-availability (*108*).

Pfizer's second generation MEK1/2 inhibitor PD0325901 has a similar structure to CI-1040 but enhanced pharmaceutical properties. However, in clinical trials this compound presented severe side effects and it's development was discontinued (*108*).

GDC-0973 (XL518) has shown strong growth inhibition effects in cell lines harbouring *KRAS* and *BRAF* mutations. *In vivo* studies have shown that a single dose of this agent is enough to inhibit ERK1/2 phosphorylation for a period of 48h. Clinical studies show that this agent is well tolerated and Phase I clinical trials are ongoing (*108, 110*).

RDEA119 (BAY869766) is a potent allosteric MEK1/2 inhibitor that efficiently inhibits growth in cell lines, and exhibits potent activity in xenograft models of melanoma, colon and epidermal carcinoma. RDEA119 is currently in Phase I trials as a single agent and in combination with Raf inhibitor Sorafenib (*108, 111*).

1.5.1 – AZD6244 (ARRY142886 / Selumetinib)

One agent that I am especially interested in is the MEK1/2 inhibitor AZD6244 (ARRY142886 / Selumetinib). In particular, I wish to investigate the response to treatment with this agent using human colorectal tumour explants, and identify markers of sensitivity / resistance that will allow for the selection of those patients more likely to benefit from treatment with AZD6244.

The MEK1/2 inhibitor AZD6244 developed by AstraZeneca is a highly selective non allosteric and non ATP competitive compound with nanomolar activity against purified MEK1/2 enzyme (*112, 113*). The molecular structure of AZD6244 is

shown in Figure 1.4. This agent strongly inhibits ERK1/2 phosphorylation shortly after exposure in cell lines and xenograft models (*94, 114*) and in lymphocytes from 12-*O*-tetradecanoylphorbol-13-acetate-treated whole blood used as a surrogate for tumour tissue in clinical trials (*115*). AZD6244 induces cell cycle arrest at G1 phase in cell lines, evidenced by a decrease in Cyclin D1 and CDK4 expression, and an increase of cell cycle inhibitors p21 and p27 (*95*). However, sensitivity to this agent is variable and induction of apoptosis is rarely reported. The mutational status of *KRAS* and *BRAF* has been proposed as an indicator of sensitivity to AZD6244. However, different authors report opposite observations. While Davies *et al* (*94*) report that cells carrying these mutations are more sensitive to the drug, Balmanno *et al* (*114*) have demonstrated that mutational status of *KRAS* or *BRAF* cannot be used as an indicator of sensitivity to AZD6244 in colon cancer cell lines.

AZD6244 has completed Phase I and II clinical trials with no severe side effects being associated with its intake, but disease stabilisation is generally reported as the best outcome (*115-118*).

AZD6244 has also shown promise when used in combination with other agents such as Akt, PI3K or mTOR inhibitors (*119-121*). Therefore, I wish to test the potential of therapeutic combinations of AZD6244 and aspirin, as several reports indicate that the pathways targeted by these drugs have some common substrates (See Chapter 6).

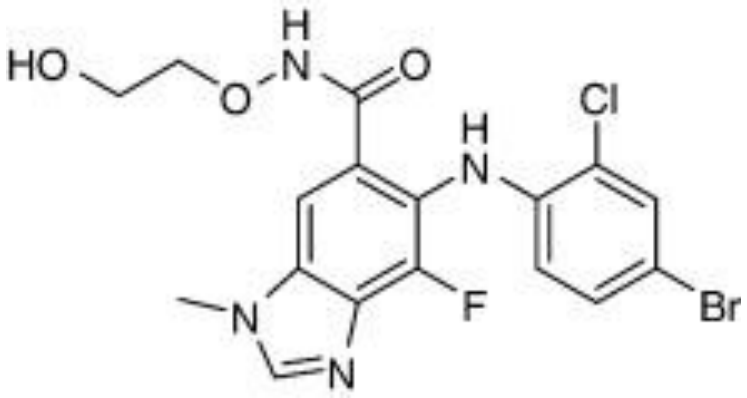


Figure 1.4: Schematic representation of AZD6244 chemical structure. Chemical structure of 6-(4-Bromo-2-chloro-phenylamino)-7-fluoro-3-methyl-3H-benzoimidazole-5-carboxylic acid (2-hydroxy-ethoxy)-amide .Adapted from Wang *et al* (*112*).

1.6 – Aspirin

Aspirin or acetylsalicylic acid is a Non Steroidal Anti Inflammatory (NSAID) drug commonly used to relieve mild pain, fever and inflammation. Regular aspirin intake is long known to prevent cardiovascular disease (*122, 123*) and this agent is routinely prescribed to patients for the treatment and prevention of this condition. Recent reports have also shown evidence that regular aspirin intake reduces the risk of colorectal cancer (*124, 125*). However, aspirin intake is associated with serious gastrointestinal and renal side effects (*126*).

Despite extensive research, the mechanisms of action of this drug are still elusive, but aspirin seems to affect a variety of cellular functions. Aspirin is a known COX inhibitor but reports show that it can also induce COX-2 expression in the gastrointestinal tract (*127, 128*). COX enzymes 1 and 2 are almost identical in structure but have evolved distinct functions. COX-1 is involved in the synthesis of protective prostaglandins that preserve integrity of intestinal lining and normal renal function, while COX-2 is involved in the inflammatory response and cell growth control (*129*).

The NF-κB pathway regulates the transcription of many genes involved in the immune and inflammatory response (*130*), and is thought to promote cell proliferation and inhibit apoptosis (*131*). It has been hypothesised that the activation of this pathway contributes to the initiation and progression of cancer by inducing cellular transformation and proliferation, preventing elimination of pre-neoplastic and malignant cells, and up-regulating anti-apoptotic signals. NF-κB pathway is also thought to promote angiogenesis and the development of metastasis (*132*). The host lab has identified a novel mechanism for switching off NFκB activity in colorectal

cancer cells. In this model, aspirin activates the NF-κB pathway through degradation of IκB which results in nuclear translocation of NF-κB and nucleolar sequestration of RelA leading to reduced transcription of anti-apoptotic signals (*133, 134*). Treatment with aspirin also induces Cyclin D1 degradation and cell cycle arrest in colorectal cancer cell lines (*135*).

Recent evidence also shows that aspirin inhibits mTOR, activates AMPK, and induces autophagy in colorectal cancer cells (*136*). The mammalian target of rapamycin, mTOR, plays a fundamental role in various signalling pathways deregulated in cancer (*137*) while AMPK, a cellular energy sensor, responds to cellular stress by suppressing cellular growth through inhibition of the mTOR pathway (*138*).

Though evidence shows that aspirin is a potent anti-cancer agent, its use is limited by the presence of side effects. It is therefore essential to unravel the exact mechanisms through which aspirin acts on cancer cells so that other drugs that mimic these mechanisms but with reduced side effects can be developed.

In order to study these mechanism as well as the mechanisms of response to MEK1/2 inhibitor AZD6244, and the potential for combination therapy with aspirin, a model of human colorectal cancer that is more relevant to the clinic should be used.

1.7 – Models of Colorectal Cancer

Several models of colorectal cancer have been developed, but so far, very few have been able to recapitulate the tumour's *in vivo* environment and original characteristics, and therefore accurately represent the variability of human disease. Ideally, study models should be able to reproducibly mimic the cell-cell and cell-

stroma interactions, reflect the invasive behaviour of tumour cells *in vivo*, and enable the study of the different mechanisms of tumour biology (*139*). However, replicating the immune component of colorectal cancers still presents a considerable challenge even in models capable of meeting the above criteria.

1.7.1 – Cell line models

Cell line models, which have been obtained from primary culture of isolated human colonic tumour cells or from immortalised normal epithelial cells, provide useful and widely used tools to unravel signalling pathways involved in colorectal cancer and to quickly obtain drug response results. However, these models are often two-dimensional (2D) systems that do not accurately mimic the structure, function and physiology of the original tissue (*140*). Even in three-dimensional (3D) systems, where the original tissue architecture is thought to be maintained, the distribution of oxygen, nutrients, and signalling molecules; and cell-cell and cell-matrix interactions, are not representative of the tissue's *in vivo* environment (*141*). Cell culture models are also limited by the genotypic and phenotypic changes that result from the immortalisation process and continuous passages, which makes them less representative of the tissue of origin (*142*).

1.7.2 – Murine models

Murine models of colorectal cancer have been invaluable tools for studying the events involved in the initiation and progression of this disease. The most widely used murine model is the multiple intestinal neoplasia mouse or $Apc^{min/+}$ mouse.

Apc$^{min/+}$ mice are heterozygous for the *Apc* gene, carrying one mutated and one functional copy of this tumour suppressor gene. While mice carrying homozygous *Apc* mutations die during embryonic stage, mice with heterozygous mutations develop multiple intestinal tumours within weeks of birth providing a study model of the early stages of carcinogenesis (*143*). Though loss of APC is considered an initiating event in the development of colorectal cancer, mutations in other genes are needed for tumours to progress towards malignant disease. Modifications of the Min mouse obtained by interbreeding with mice carrying mutations in other genes like *KRAS, p53* or members of the Wnt pathway have also been developed to study the mechanisms underlying tumour progression (*144*).

Even though *Apc*$^{min/+}$ mice are an important tool for studying the events involved in the initiation of colorectal cancer, the model has limitations. *Apc*$^{min/+}$ mice develop polyps mainly in the small intestine while in humans polyps appear mainly in the colon. *Apc*$^{min/+}$ mice rarely present invasive adenocarcinomas, and the presence of metastasis has never been reported, making them inadequate for the study of later stages of the disease (*145*). Even modifications to the *Apc*$^{min/+}$ mouse which can display more aggressive tumours are limited by differences in phenotype between mice and human disease. Also, *Apc*$^{min/+}$ mice predisposition to colorectal cancer is strain dependent and regulated by a genetic loci designated as Modifiers of Min or *Mom*. To date a variety of *Mom* loci have been identified. Based on this, *Apc*$^{min/+}$ mouse strain and breeding programmes need to be carefully chosen for each specific experiment (*146, 147*).

Other genetically engineered mouse models have been developed in which mutations in genes involved in the initiation and progression of colorectal cancer are introduced. However, manipulation of these genes in mice does not completely recreate the phenotype observed in the human disease, and it may not represent the mechanisms behind sporadic colorectal cancer. Also, in such models, genetic manipulation is mostly based on loss of function while in human cancers many mutations lead to gain of function (*148*). Genetically engineered mice also display considerable variability in terms of tumour development regarding frequency, growth rate and tumour location caused by modifier alleles characteristic of each individual, that can significantly influence tumour initiation or response to therapeutic agents (*149*).

Transplantation or xenograft models are a common tool for studying metastatic colorectal cancer, and therapeutic responses. In this model, human cancer cell lines or fragments of human tumours are transplanted into immuno-compromised mice where they form tumours if cells are used, or continue to develop in the case of tumour fragments, become invasive and eventually form metastasis (*150*). However, the formation of metastasis is a lengthy process that occurs only in a small number of animals. Xenografts are also often not representative of the genetic and histological characteristics of the human tumours, and thus not entirely predictive of therapeutic response (*149*). In addition, the use of immuno-compromised mice does not allow for assessment of the role of the immune component in tumourigenesis (*151*). Hence, humanised mice have recently become a popular solution to overcome this limitation. Mice humanisation techniques include ablation of the mouse immune system followed by engraftment of human immune

cells (*152*), transgenic expression of human immunoreceptor genes (*153*), or the use of recombinase mediated genomic replacement through which human loci for the entire T-cell and B-cell receptors or even major specific compatibility complexes might be knocked in in the mice. However, these techniques also present technical and data interpretation limitations as any modifications introduced in the models must be taken into account when analysing the data (*151*).

Inflammation-mediated mouse models have been used to show that chronic inflammation can develop into colorectal cancer. In these studies, mice were given a single dose of Azoxymethane (AOM) followed by prolonged treatment with dextran sulphate sodium (DSS) which resulted in chronic colitis, high-grade dysplasia and consequent progression to colorectal cancer. However, multiple treatments with carcinogens during long experimental periods are necessary to induce large tumours. Also, these mice develop invasive colonic carcinomas with *ras* mutations but rarely present *Apc* or *p53* mutations (*154*), and therefore lack fundamental characteristics of the human disease.

Other murine models have been developed to study different aspects of colorectal cancer like carcinogen induced models to study sporadic colorectal cancer; *Tgfb1* mutant mice to study later stages of the disease; or mismatch repair (MMR)-deficient models to study Hereditary Non Polyposis Colorectal Cancer (*147, 155*). Despite being fundamental tools to study different aspects of colorectal cancer, murine models are often driven by specific mutations and therefore present characteristic aetiology that is distinct from the human colorectal cancers. These

models also do not take into account the phenotypic differences between mice and human disease, which limits direct extrapolation from one to the other.

1.7.3 – Multicellular tumour spheroid models

Multicellular tumour spheroids are 3D aggregates of tumour cells or of a mixture of tumour and stromal cells that can be obtained from a broad range of human tumour cell lines or from tissue previously subjected to disaggregation techniques, with the former being more common. This model, initially used to study tumour radiobiology is still mostly applied in therapeutic response studies, though some investigators have also used it to study basic biological mechanisms (*156, 157*). Being 3D structures, multicellular tumour spheroids can recreate the complex mechanisms of continuous cell-cell and cell-matrix interactions present in living tissues, and can also present various pathophysiological features of avascular tumours and developing metastasis (*139, 158*).

There are several methods of obtaining multicellular tumour spheroids but the main requirement of this technique is that the cells, either cell lines or cells disaggregated from tissues *ex vivo*, attach to each other or to an artificial matrix in order to form the 3D structure. The spinner flask method, with which cells are grown as a suspension culture in liquid medium with constant agitation to impede the adhesion of the cells to the flask wall and instead promote the adhesion between cells, is the most common method. Other alternatives are: liquid overlay cultures, microcarrier beads coated with cell binding materials (*139*), scaffold based cultures (*159*), or NASA's rotary cell culture system (*160*).

Despite their general use and numerous advantages, multicellular tumour spheroids do not fully mimic the *in vivo* environment of the original tissue, especially when cell line derived spheroids are used. In these models, it is impossible to accurately say that the behaviour of the cells in terms of growth and death will be the same as the tumour *in vivo*; firstly, because tumour cell lines are often manipulated to suit laboratory and commercial needs, and when kept in culture can accumulate mutations that confer altered characteristics. Secondly, because when using cell lines there isn't an original tumour to which the spheroid architecture can be compared to. Even spheroids obtained from disaggregated tumours might not be representative of the properties found in the original tissue due to the requirement of an extracellular matrix that, depending on its nature, can alter the cells behaviour in culture (*159*). Regardless, of such limitations human multicellular tumour spheroids obtained from disaggregated tissue are becoming increasingly more popular and their use in a variety of application has been reported by several authors (*41, 48, 161, 162*).

The culture of multicellular spheroids of normal intestinal tissue has also been reported by several authors (*35, 163, 164*). In a recent publication, Sato *et al* (*163*) described a method for the long term culture of organoids derived from human intestinal tissue obtained from surgery. The authors reported how, by adding nicotinamide, a member of the vitamin B family; A83-01, an Alk3/4/5 inhibitor; and SB202190, a p38 inhibitor to the culturing media they were able to maintain the organoids in culture for periods of more than 6 months. However, the authors also observed a lack of cell differentiation shown to be caused by the presence of nicotinamide and SB202190. The same authors also reported that the use of R-sponding1, a Lgr5 ligand essential for activation of the Wnt signal in the intestinal

crypt (*165*); and noggin, a protein involved in intestinal crypt formation and polyp growth (*166*) are fundamental for the long term maintenance of mouse intestinal adenoma organoid cultures. These culturing systems can, therefore, be used to study basic aspects of intestinal stem cell biology and cell differentiation, and to investigate response to therapeutic agents to be used in a variety of intestinal conditions.

1.7.4 – *Ex vivo* models

Ex vivo models are three dimensional culture systems of tissue explants that fill the gap between the 2 dimensional cell culture models and the use of animal models. Because they more accurately represent the 3D architecture found in the *in vivo* environment preserving cell-cell and cell-stroma interactions, they are valuable tools for studying other aspects of intestinal biology such as morphology and epithelial homeostasis, cell differentiation, organisation and biochemical responses. They are more appropriate tools for the development of new therapies, and for testing the efficacy of currently available or new therapeutic agents in a clinically relevant context.

There are three main requirements to successfully culture human tissue *ex vivo*: the presence of an extracellular matrix; the presence of interstitial fluid rich in nutrients and signalling molecules required for tissue homeostasis; and the arrangement of the cells in culture must be such as to enable appropriate cell-cell and cell-matrix interactions, and the exchange of biological effectors (*139*). Several methods for long term culture of tumour explants that include different approaches to these requirements have been described in the literature over the last 50 years. These methods allow for the maintenance of tissue architecture, homeostasis and oncogenic

properties, and can be used for a variety of applications including drug response assays (*167-169*). However, the culture of human tumour explants *ex vivo* is not widely and routinely used. Availability of fresh tissue samples can be a limiting factor, along with the ethical restrictions imposed to the use of human biopsies, and the need for patient consent.

1.8 – Project Aims

The aims of this project were:

I. To develop a method of culturing human colorectal tumour explants *ex vivo* that allows for the maintenance of the tumours individual characteristics providing a robust and representative model of human colorectal cancer.

II. Use this model to test the pharmacodynamic and phenotypic effects of the MEK1/2 inhibitor AZD6244.

III. Investigate the relationship between pharmacodynamic and phenotypic responses and known genetic factors of sensitivity / resistance to AZD6244.

IV. Use this platform to explore the potential for combination therapy with AZD6244 and aspirin.

Chapter 2: Materials and methods

2.1 - Materials and reagents

2.2.1 – Tumour explant culture

- Waymouth medium (Gibco) supplemented with foetal calf serum (10%, Gibco), penicillin/streptavidin (1.7g/mL and 1.3g/mL respectively, Gibco), ascorbic acid (300µg/mL, Sigma), FeSO4.7H20 (0.45µg/mL, Merck), antimycotic/antibiotic mix (1:100, Sigma), and hydrocortisone (3µg/mL, manufactured by Biochrom AG, supplied by Autogen Bioclear).

- MEM medium (Sigma) supplemented with foetal calf serum (10%, Gibco), penicillin/streptavidin (1.7g/mL and 1.3g/mL respectively, Gibco), antimycotic/antibiotic mix (1:100, Sigma), and L-glutamine (4mM, Sigma).

- StemPro hESC SFM stem cell medium (Gibco): DMEM/F-12 + 1x Glutamax media supplemented with 1x StemPro hESC SFM Growth supplement, 1.8% BSA, 8ng/mL FGF-basic, 0.1mM 2-Mercaptoethanol, penicillin/streptavidin (1.7g/mL and 1.3g/mL respectively, Gibco), and antimycotic/antibiotic mix (1:100, Sigma).

- FGF-basic (Invitrogen) reconstituted in 10mM Tris (pH 7.6).

- Collagen gels supplied by First Link Ltd and prepared according to manufacturer's instructions: 4.5mL of collagen gel were mixed with 0.5mL of supplemented MEM media, and pH was adjusted with 1M NaOH until solution turned from yellow to pink in colour. Gels were then plated on 24-well plates (0.5mL of gel solution per well) and allowed to set for 5min at 37ºC.

2.1.2 – Cell culture

- RPMI 1640 media (Gibco) supplemented with foetal calf serum (10%, Gibco), penicillin/streptavidin (1.7g/mL and 1.3g/mL respectively, Gibco), antimycotic/antibiotic mix (1:100, Sigma) was used to culture HRT18 cells.

- McCoy's media (Gibco) supplemented with foetal calf serum (10%, Gibco), penicillin/streptavidin (1.7g/mL and 1.3g/mL respectively, Gibco), antimycotic/antibiotic mix (1:100, Sigma) was used to culture RKO and HCT116 cells.

2.1.3 – AZD6244 and Aspirin treatments

- AZD6244 (AstraZeneca.) AZD6244 was dissolved to 10mM stock solution in DMSO. Working solutions were also dissolved in DMSO. Stock and work solutions were stored at 4ºC.
- Aspirin was obtained as acetyl salicylic acid from Sigma, and 500mM stock solutions were prepared in house by slowly adding 5M NaOH to an acetyl salicylic acid solution previously prepared with water. This solution was allowed to hydrolyse overnight and pH was adjusted to 7. Stock solution was stored at -20ºC until use.

2.1.4 – Viability assays

2.1.4.1 – MTT based in vitro toxicology assay

MTT assay used to assess viability of tumours and outgrowths while in culture was supplied by Sigma and used according to manufacturer's instructions. MTT reagent was reconstituted in 3mL of sterile PBS, aliquoted and frozen until use.

2.1.4.2 – Sulforhodamine B colorimetric cytotoxicity assay

Sulforhodamine B (SRB) was used to measure the effects of treatment with AZD6244 and aspirin, individually and in combination, in colorectal cancer cell lines.

- 25% Trichloroacetic acid solution: trichloroacetic acid (Sigma) was dissolved in water to a concentration of 25% and stored at 4ºC.

- 1% acetic acid: acetic acid (Sigma) was diluted to a concentration of 1% with water immediately before use.

- 0.4% SRB in 1% acetic acid: sulforhodamine B was dissolved in 1% acetic acid to a concentration of 0.4% and stored at 4ºC.

- 10mM Tris pH10.5: Tris base (Sigma) was dissolved in water to a concentration of 10mM and pH was adjusted to 10.5. Solution was stored at 4ºC.

2.1.5 – Immunoperoxidase assay

- TEG Buffer: 2.422g Tris and 0.38g EGTA dissolved in 2L of distilled water, and pH adjusted to 9.

- Wash Solution 1 - 1% BSA (Sigma) + 0.2% Gelatine (Sigma) + 0.05% Saponin (Sigma): 2g of gelatine were added to 200mL PBS and heated in the microwave until dissolved. Gelatine was then added to 10g BSA and 0.5g saponin previously dissolved in PBS. 1L of this solution was prepared.

- Wash Solution 2 – 0.1% BSA (Sigma) + 0.2% Gelatine (Sigma) + 0.05% Saponin (Sigma): 4g of gelatine were added to 400mL PBS and heated in the microwave until dissolved. Gelatine was then added to 2g BSA and 1g saponin previously dissolved in PBS. 2L of this solution were prepared.

2.2 – Methods

2.2.1 – Tumour explant culture

Tumours were collected at the time of resection and immediately transferred to the lab immersed in culturing media. Tumours were placed in Falcon tubes containing sterile Phosphate Buffered Saline (PBS), washed twice by inverting the tubes, and then placed in Petri dishes containing sterile culturing media where they were dissected into 1-2mm^2 fragments. Tumour fragments were washed once more in new Petri dishes containing fresh media and cultured using the methods described

below. Prior to placing in culture, a proportion of the tumours was routinely frozen and a proportion fixed as a T0 control.

2.2.1.1 – Growth on stainless steel mesh

Tumour fragments were inserted in cuts made on the edges of the stainless steel mesh, and these were balanced on the centre well of a culturing dish containing 3mL of Waymouth media (Gibco) supplemented with foetal calf serum (10%, Gibco), penicillin/streptavidin (1.7g/mL and 1.3g/mL respectively, Gibco), ascorbic acid (300µg/mL, Sigma), FeSO4.7H20 (0.45µg/mL, Merck), antimycotic/antibiotic mix (1:100, Sigma), and hydrocortisone (3µg/mL, manufactured by Biochrom AG, supplied by Autogen Bioclear). Cultures were then placed in a Molecular Incubator Chamber (billups-rothenberg Inc.) pressurised for 3min with 95% O_2 and 5% CO_2, and incubated at 37°C with gentle rocking. Tumours explants were transferred to new culture dishes containing fresh media, daily. Tumours were harvested after 4 days in culture, and frozen or fixed in 4% Formaldehyde/PBS.

2.2.1.2 – Unattached growth

Tumour fragments were immersed in 3mL of supplemented Waymouth media in the centre well of the culturing dish. One fragment per dish was set up. Tumours were cultured in a molecular incubator chamber pressurised for 3min with 95% O_2 and 5% CO_2, and incubated at 37°C with gentle rocking. Daily media and dish changes were performed. Samples were harvested at T0, 1, 5, 10, 16, and 20 days, frozen or fixed in 4% formaldehyde.

2.2.1.3 – Growth on filter paper

Tumour fragments were individually placed on filter paper disks previously soaked in supplemented Waymouth media, and transferred to the centre well of a culturing dish containing 3mL of fresh media. Media and culture dishes were replaced daily. Explants were cultured in a molecular incubator chamber pressurised for 3min 95% O_2 and 5% CO_2, and incubated at 37°C with gentle rocking, and harvested at T0, 1, 5, 10, and 16 days,. Samples were frozen or fixed in 4% formaldehyde.

2.2.1.4 – Growth on collagen gels

Tumours previously washed and dissected into 1-2mm^2 fragments were washed a third time in fresh MEM media (Sigma) supplemented with foetal calf serum (10%, Gibco), penicillin/streptavidin (1.7g/mL and 1.3g/mL respectively, Gibco), antimycotic/antibiotic mix (1:100, Sigma), and L-glutamine (4mM, Sigma) before plating on Type I collagen gels (First Link Ltd) previously prepared according to manufacturer's instructions and allowed to set in 24-well plates. Samples were covered with 200µL of supplemented media and incubated at 37°C with 5% CO_2. Fresh media was added to the cultures every 2 days. Samples were harvested at T0, 24/48h, 5, 7, 12, 16 and 28 days. Main tumour bodies were immediately frozen or fixed in 4% formaldehyde. Cell outgrowths were digested with Collagenase IV as described below. Cultures were imaged, immediately before harvest, using a Nikon AZ100 macroscope with 1x magnification objective to monitor the progress of the explants while in the culture and the formation of outgrowths.

2.2.1.4.1 - Digestion with collagenase IV

Collagenase IV enzyme (200U/mL, Sigma) was solubilised in supplemented MEM media and filtered with a 40μm syringe filter before use. Collagen gels, outgrown cells and media were removed from the culture wells and placed in Falcon tubes to which the collagenase solution was added. This mixture was incubated at 37°C for 45min then centrifuged at 500g for 10min at 4°C. Cell pellets were washed with cold PBS solution by centrifugation at 1000g for 5min at 4°C. PBS was discarded and pellets were immediately stored at -80°C.

2.2.1.5 – Preparation of multicellular tumour spheroids

Tumours collected at the time of resection were immediately transferred to the lab immersed in culturing media. Once in the lab, tumours were washed twice in sterile PBS, placed in Petri dishes containing fresh PBS and dissected into $1mm^2$ fragments. Tumour fragments and PBS were transferred to Falcon tubes, washed by inverting the tubes, and centrifuged at 1000rpm for 5min at 4ºC. Tumours were washed 3x following this procedure, and re-suspended in supplemented MEM media containing 1.6mg/mL Collagenase IV (Sigma). Fragments were mechanically disrupted by pipetting up and down with a 5mL pipette, then transferred to Erlenmeyer flasks (Fisher Scientific) and incubated at 37ºC for 2h with vigorous rocking. Digested tumours were transferred to falcon tubes and centrifuged at 1000rpm for 5min at 4ºC. Pellets were re-suspended in PBS, washed by inverting the tubes, and filtered through a 500μm stainless steel mesh. Filtrate was again passed through a 40μm cell strainer. This filtrate was discarded and tumour fragments that

remained in the strainer were collected onto a Falcon tube and centrifuged at 1000rpm for 5min at 4ºC. Pellets were re-suspended in StemPro hESC SFM stem cell media (Gibco) which is composed of DMEM/F-12 + 1x Glutamax media supplemented with 1x StemPro hESC SFM Growth supplement, 1.8% BSA, 8ng/mL FGF-basic, 0.1mM 2-Mercaptoethanol, penicillin/streptavidin (1.7g/mL and 1.3g/mL respectively, Gibco), and antimycotic/antibiotic mix (1:100, Sigma). Tumour fragment suspension was transferred to 6-well plates and incubated at 37ºC with 5% CO_2. Formation of multicellular tumour spheroids was monitored daily by imaging with a Zeiss LSM510 confocal microscope.

2.2.2 – Treatment of colorectal tumour explants

All colorectal tumour explant culture treatments were performed with culturing media supplemented with 10% foetal calf serum, as described below.

2.2.2.1 – Long term treatment

Tumour fragments were placed on collagen gels and covered with 200µL of supplemented MEM media containing 0, 1, 3, 5 and 10mM aspirin and cultured for 24h or 3-6 days or 0, 1, 3, 5 and 10µM oxaliplatin (Sigma), and cultured for 24 and 48h without media or drug replenishment.

2.2.2.2 – Acute (short time point) treatment

Colorectal tumours collected at the time of resection were immediately transferred to the lab immersed in culturing media. Tumours were placed in Falcon

tubes containing sterile PBS, washed twice by inverting the tubes, and then placed in Petri dishes containing sterile culturing media and dissected into 1-2mm^2 fragments. Tumours were transferred to new Petri dishes and washed with fresh media before plating. Treatment of tumour explants was performed in 96-well plates and in the presence of 10% foetal calf serum as described below:

AZD6244 treatments: tumours were individually immersed in 200µL of supplemented MEM media containing 0, 0.1 or 3µM AZD6244, and routinely incubated for 1h at 37ºC with 5% CO_2. The first 3 tumours treated were incubated for 1, 3 and 6h as part of an experiment aiming to determine the minimum time point at which the effects of AZD6244 could be measured. DMSO concentration was 0.03% in all wells, including non treated controls (0µM).

Treatment with combinations of AZD6244 and Aspirin: tumours were individually immersed in 200µL of supplemented MEM media containing:

- 0µM AZD6244 + 0.03% DMSO
- 0.1µM AZD6244 (final DMSO concentration 0.03%)
- 3µM AZD6244 (final DMSO concentration 0.03%)
- 0.1µM AZD6244 + 100µM aspirin (final DMSO concentration 0.03%)
- 3µM AZD6244 + 100µM aspirin (final DMSO concentration 0.03%)
- 0µM aspirin
- 100µM aspirin

Tumour explants were incubated for 1h at 37ºC with 5% CO_2.

2.2.3 – Cell culture

Colorectal cancer cell lines RKO, HRT18 and HCT116 (American type culture collection) were selected for these assays based on their reported sensitivity to AZD6244 and mutational status of *KRAS* and *BRAF*. RKO cells are *BRAF* mutant while HRT18 and HCT116 are *KRAS* mutant.

All cell lines were grown in T75 flasks and split every 3 days into new flasks with fresh media. HRT18 cells were maintained in RPMI 1640 media (Gibco) supplemented with foetal calf serum (10%, Gibco), penicillin/streptavidin (1.7g/mL and 1.3g/mL respectively, Gibco), antimycotic/antibiotic mix (1:100, Sigma). RKO and HCT116 cells were maintained in McCoy's media (Gibco) supplemented with foetal calf serum (10%, Gibco), penicillin/streptavidin (1.7g/mL and 1.3g/mL respectively, Gibco), antimycotic/antibiotic mix (1:100, Sigma).

2.2.3.1 – Treatment with AZD6244

RKO, HRT18 and HCT116 were split into T25 flasks or 6-well plates containing glass cover slips, and allowed to grow in supplemented culturing media (see materials and reagents) for 24h before treatment to assure complete adhesion and ~60% cell confluence. Culturing media was then replaced with fresh media containing 0, 0.1 or 3μM AZD6244. Cells were incubated for 1, 3 and 6h at 37ºC with 5% CO_2. Cells grown on T25 flasks were washed with PBS and transferred to 15mL Falcon tubes and centrifuged. Cell pellets were frozen for protein extraction (see section 2.2.4.4). Cells grown on cover slips in 6-well plates were washed with PBS and fixed in Methanol / Acetone (1:1) solution for a minimum of 30min. at 4ºC,

and immunocytochemical analysis for relevant markers was performed (see section 2.2.4.3).

2.2.3.2 – Treatment with combinations of AZD6244 and Aspirin

Prior to treatment with drug combinations growth curves for each cell line were established to determine the optimal plating density and time point at which cells achieved exponential growth. Half maximum effective concentrations ($EC_{50)}$ for each drug and cell line were also determined

2.2.3.2.1 – Establishment of growth curves

Cells were split and plated in seven 96-well plates at increasing cell densities and allowed to grow for 7 days. Six replicates were set up per original plating density and assays were performed in triplicate for each cell line. Cells were harvested every day (one 96-well plate per day) and fixed. Cell viability was assessed using a Sulforhodamine B assay (see section 2.2.4.5.2). Growth curves were generated from the absorbance values.

2.2.3.2.2 – EC_{50} calculation

Cells were plated at densities previously established using the growth curves and allowed to grow for 1 (HRT18 and HCT116 cells) or 2 days (RKO cells) until exponential growth was achieved. Culturing media was then replaced by fresh media containing increasing concentrations of AZD6244 or aspirin. Six replicates were

used for each drug concentration. Cells were treated for 72h (no replenishment of drug or media), fixed and assessed for viability using a Sulforhodamine B assay. Absorbance values generated from this assay were used to calculate EC_{50} values using Prism software. Assays were performed in experimental triplicates for each drug and cell line.

2.2.3.2.3 – Drug combination assays

Cells were plated in 96-well plates at densities previously established and allowed to grow for 1 day (HRT18 and HCT116 cells) or 2 days (RKO cells) until exponential growth was achieved. Culturing media was then replaced by fresh media containing increasing concentrations of AZD6244 and aspirin. Drug concentrations were established based on the EC_{50} values previously determined for each drug. Four dilutions below and 3 dilutions above the EC_{50} values were used for each drug, and the range of concentrations used varied with cell line. RKO cells were treated with AZD6244 concentrations ranging from 0-70μM and aspirin concentrations ranging from 0-15mM. HRT18 cells were treated with AZD6244 concentrations ranging from 0-135μM and aspirin concentrations ranging from 0-25mM. HCT116 cells were treated with AZD6244 concentrations ranging from 0-38μM and aspirin concentrations ranging from 0-27mM. Six replicates of each treatment were set up per plate and assays were performed in triplicate for each cell line.

Cells were treated for 72h without replenishment of media or drugs. Cell viability was assessed using a Sulforhodamine B assay. Absorbance values obtained with this assay were then used to calculate the percentage of cell death as a result of

treatment with each drug individually or in combination. The effects of the drug combinations were determined using Compusyn software.

2.2.4 – Assays

2.2.4.1 – Fixation, Embedding and Staining of tumour samples

Samples were fixed in 4% formaldehyde/PBS for 24h then immersed in 75% ethanol for 24h, and paraffin embedded using a Sakura Tissue-Tek VIP 5 Jr machine. 4μm tissue sections were cut using a Reichert-Jung Biocut 2030 and placed on Superfrost Plus slides (Thermo Scientific) for histological and immunohistochemical analysis.

2.2.4.1.1 - Haematoxylin & Eosin staining

Formaldehyde fixed and paraffin embedded (FPPE) tissue sections were de-waxed in xylene and re-hydrated by serial washes in decreasing concentrations of ethanol, and finally in water. Slides were immersed in haematoxylin for nuclear staining, differentiated in acid alcohol (1% HCl in 70% alcohol), immersed in lithium carbonate to remove excess dye and then in eosin for cytoplasm differentiation. Slides were then dehydrated in a series of washes in absolute ethanol and xylene, mounted in D.P.X. mounting medium (BDH), and allowed to dry for 24h before imaging.

2.2.4.1.2 – Immunofluorescence assay

FPPE tissue sections were placed in the oven at 60°C for 1 ½ hours, de-waxed in two xylene washes, and hydrated by serial washes in absolute ethanol, 70% ethanol and single distilled water. Antigen retrieval was performed by boiling the slides in 10mM citrate buffer (pH6) for 25min. Slides were then incubated overnight at 4ºC in a solution of 10% Donkey Serum (Sigma), 5% Bovine Serum Albumin (Sigma) and 1% Tween diluted in PBS, for non-specific background blocking. Blocked slides were washed with PBS and probed with primary antibodies against PCNA (1:100, Santa Cruz Biotechnology), FITC-Ki-67 (1:50, Dako) or active caspase-3 (1:100, BD Bioscience). After a 4h incubation at room temperature slides were again washed in PBS and correspondent secondary antibodies were added to each slide (0.5:1000, Jackson ImmunoResearch Laboratories). Secondary antibody incubation was performed at room temperature for 25 minutes, after which slides were washed with 1% Tween/PBS. Slides were then incubated with 1µg/mL DAPI/PBS (Invitrogen) for nuclear staining, washed with PBS, mounted with Vectashield mounting medium (Vector Laboratories), and imaged (see section 2.2.4.5 for details).

2.2.4.1.3 – Immunoperoxidase assay

FPPE tissue sections were placed in the oven at 60°C for 1 ½ hours, de-waxed in serial washes of xylene, absolute ethanol and 96% ethanol, and immersed in a solution of 30% H_2O_2 / methanol for 30min to block endogenous peroxidase. Sections were then hydrated in serial washes of 70% ethanol and water, and boiled in TEG buffer for 30min. Slides were allowed to cool at room temperature for 30min

and then incubated in 50mM NH_4Cl for 30min at room temperature. Sections were washed with Wash Solution 1 (see section 2.1.5) and probed with KRT18 antibody (1:200, Sigma) overnight at 4ºC. After primary antibody incubation sections were washed with Wash Solution 2 (see section 2.1.5) and probed with correspondent secondary antibody (1:200, Promega) for 30min at room temperature. Slides were washed again with Wash Solution 2 and stained with DAB (Sigma) at room temperature for 10min. Slides were then washed with PBS and water, counterstained with haematoxylin and rinsed in water. Excess die was removed by washing in saturated lithium carbonate solution and again in running water. Sections were then dehydrated in serial washes of absolute ethanol and xylene, mounted with D.P.X. mounting medium (BDH) and allowed to dry overnight before imaging.

2.2.4.2 – Imaging and analysis

Brightfield microscopy was performed with Zeiss Axioplan 2 microscope with 20x or 40x objectives.

Fluorescent microscopy was performed with a Zeiss Axioplan 2 microscope with 40x or 100x Plan Neofluor objectives and a Chroma 83000 filter set. Each channel was recorded independently, and pseudocolor images were superimposed. Images were captured using in-house scripts written for IPLab software.

Five fields of view per marker per sample were captured “blindly” to remove bias. For each marker, the total number of cells and the number of positive cells was counted, the percentage of positive cells per field was calculated and the average of the five fields determined.

Epithelial cell count was performed on 400x magnification fields of view, while quantification of proliferation and apoptosis markers was performed on 1000x magnification fields of view.

2.2.4.3 – Immunocytochemistry

Cells grown on cover slips and previously fixed were washed with PBS and incubated for 30min at room temperature in a solution of 10% donkey serum in PBS to block non-specific protein. After incubation this solution was discarded and replaced by primary antibodies diluted to 1:100 in 10% donkey serum solution previously used. Primary antibody incubation was performed at room temperature for 2h. Cells were then washed with PBS and correspondent secondary antibody diluted to 1:200 in 1.5% donkey serum/PBS solution was added to the wells. Secondary antibodies were incubated at room temperature for 30min, after which cells were washed in PBS. A solution of 1μg/mL DAPI/PBS was then added to the cells and incubated for 5min. Cells were washed again with PBS and slides were mounted with Vectashield mounting medium (Vector Laboratories) and imaged as described below.

The following primary antibodies were used in this assay: Ki-67 (1:100, Dako) or active caspase-3 (1:100, BD Bioscience).

2.2.4.3.1 – Imaging and analysis

Fluorescent microscopy was performed as described in section 2.2.4.2 using a 65x Plan Neofluor objective.

Ten fields of view per marker per sample were captured "blindly" to remove bias, at a magnification of 650x. For each marker, the total number of cells and the number of positive cells was quantified, then the percentage of positive cells per field was calculated and the average of the ten fields determined.

2.2.4.4 – Western Blot analysis

To generate whole cell lysates, cell pellets, collagenase IV digested tumour pellets or frozen tumour samples were homogenised and incubated in 1x Lysis Buffer (Cell Signalling) supplemented with Complete Protease Inhibitor (40µL/mL), Pefabloc (1mg/mL), Pepstatin A (1µg/mL), NaF (1mM), Na_3VO_4 (1mM) and PMSF (1mM) for 30min with vortexing every 10min, then centrifuged at 13000rpm for 10min at 4°C. Supernatant was transferred to fresh tubes and pellets were discarded. Protein concentration was quantified using the Bio-Rad Protein Assay (Bio-Rad) according to manufacturer's instructions. 30µg of protein was loaded on to 10% polyacrylamide gels then proteins separated by SDS-PAGE at 160V for ~1½h. Proteins were transferred to Polyvinylidene fluoride (PVDF) membranes using a semidry blotter (Bio-Rad) at 10V for 30min. Membranes were blocked in 5% milk/PBS for 2h at room temperature or 45min at 37ºC, and probed with primary antibody diluted in PBS/BSA/Azide solution. Primary antibody incubation was performed at 4°C overnight or at room temperature for 1h, depending on manufacturer's instructions. After incubation, membranes were washed in PBS with 1% Tween, and probed with correspondent secondary antibodies for 1h at room temperature. Membranes were finally washed in PBS with 1% Tween and

Amersham Hyperfilm ECL films (GE Healthcare) were exposed to the blots for 1-10min and developed using a Konica-Minolta SRX101A machine.

Antibodies used for western blotting were: p-p44/42 MAPK (1:1000; Cell Signalling), p44/42 MAPK (1:1000; Cell Signaling), p-Akt (1:500; Cell Signaling), Akt (1:500; Cell Signaling), p-MEK (1:500, Cell Signaling), MEK (1:500; Cell Signalling), p27 (1:100, Calbiochem), CDK4 (1:1000; Cell Signalling), Cyclin D1 (1:200, Thermo), p21 (1:200, Santa Cruz), CDK1 (1:500, Sigma), CyclinB1 (1:500, Cell Signalling) acetylated lysine (1:500, Cell Signalling), Bcl-xS/L (1:200, Santa Cruz), TIF1A (1:1000, Assay Biotech) or β-actin (1:2000, Sigma).

2.2.4.4.1 – Western Blot quantification

Western Blot quantification was performed using ImageJ software. Band and background intensities were measured then band intensity calculated by subtracting background values from band values. All protein intensities were normalised using β-actin to correct for variability in protein loading. Phosphorylated protein was also normalised using corresponding native protein values.

2.2.4.5 – Viability assays

2.2.4.5.1 – MTT Viability Assay

MTT viability assays were performed on tumours and outgrowths cultured on collagen gels. 20μL of MTT reagent was added to each tumour culture and plates were incubated at 37ºC for 4h. The formation of purple formazan crystals indicated

viability of the tumours and cell bodies. Cultures were imaged with a Nikon AZ100 macroscope with 1x magnification objective.

2.2.4.5.2 – Sulforhodamine B viability (SRB) assay

Sulforhodamine B viability assay was performed on colorectal cancer cell lines to determine cell growth curves, establish EC_{50} values and evaluate the effects of AZD6244 and aspirin individually and in combination.

Cells were fixed in 4% TCA for 45min at 4ºC and washed 10x with water and dried at room temperature. SRB solution was added to the plates and cells were incubated at room temperature for 30min. SRB solution was removed and cells were washed 4x with 1% acetic acid, and dried. SRB was then solubilised with a 10mM Tris solution (pH10.5), and absorbance was measured at 540nm with a Bio-Tek Synergy HT plate reader and Gen 5 software.

Cell viability was determined from the average absorbance values obtained from 6 experimental repeats performed per assay. As this assay gives an indication on the levels of live cells, inverted average absorbance values were used in the AZD6244 and aspirin combination treatments to determine the level of cell death as a result of treatment.

Chapter 3: *Ex vivo* models of human colorectal cancer

Introduction:

Cancer models play a fundamental role in understanding the processes involved in cancer initiation and progression, and are vital instruments for the development and testing of new therapeutic agents. However, it is becoming increasingly clear that results obtained with the most commonly used cell based and animal models of colon cancer don't always translate to the clinic. Therefore, there is a pressing need to develop tumour models that are relevant to the human disease. An ideal model would maintain the tissue organisation and structure, oncogenic properties, differentiation functions and cellular heterogeneity presented *in vivo* (*168*). *Ex vivo* explant cultures are the only models likely to meet these requirements.

Literature reporting methods for culturing organ explants dates from the beginning of the 20th century when Carrel, in 1912, first reported maintaining embryonic heart tissue in culture for a period of 3 months (*170*). Further reports were later on presented by several authors (*171-173*). However, organ culture remained limited to embryonic tissue, possibly because that was the interest of the authors. This changed in 1959 when Trowell described a method that allowed the "satisfactory histological preservation" of explants from a variety of adult organs for 6-9 days (*174*). Despite his success in culturing tissue of various origins, he makes no reference to the culture of intestinal explants. These seemed to present increased limitations probably due to the complexity of the intestinal morphology and physiology, and the possibility of contamination. The culture of normal intestinal epithelia was first described by Browning *et al* (*175*) and later on optimised by Autrup *et al* (*176, 177*) who was able to successfully maintain normal intestinal mucosa in culture for a period of up to 28 days. To date, other methods have been

described in the literature, that are essentially, variations of the protocols described by these authors, but including different types of tissue support, such as stainless steel mesh, lens or filter paper or Gelfoam sponges, and modifications to the culturing media.

Like explant culture of normal tissues, culture of tumour tissues *ex vivo* has evolved slowly. The culture of neoplastic lesions as organs was first reported by Elias *et al* (*178*) who, by culturing normal, pre-neoplastic and neoplastic tissue from mouse mammary glands discovered that different hormonal supplements were required by normal and tumour specimens. Their work demonstrated the importance of tumour explant cultures for the study of cancer biology, development and progression. In the 1980's, Freeman *et al* described a method to culture human tumour explants of various origins, including colorectal tumours. The method which involved the use of collagen gels for tissue support and media supplemented with amino acids and antibiotics, allowed for tumour growth for a period of up to 28 days and maintenance of morphological and homeostatic properties (*168*). This method was later on successfully used by the group to study the response of explants from different tumour types to an array of therapeutic agents. Their results indicated that the method could be used to predict response in the clinic (*169*). More recently, similar methods of culturing human tumours, including colorectal explants that use collagen gels or Gelfoam sponges for tissue support and variations to the culturing media initially used by Freeman *et al* have been described in the literature. These methods can be used in a variety of applications such as the study of tumour biology or response to therapeutic agents.

Though scientists have been attempting to grow normal colonic mucosa and colonic tumours as organ cultures since the 1960's, the protocols described have not been widely used. The growth of both normal and malignant tissue *ex vivo* is technically and financially demanding, and frequently limited by the availability of patient material and associated ethical requirements. However, *ex vivo* models of colorectal cancer present numerous advantages when compared to cell based and animal models, the most important being the maintenance of the tumour's individual characteristics and cellular heterogeneity. An *ex vivo* model of colorectal tumours will enable study of the response to therapeutic agents, highlighting pathways and molecular markers of sensitivity and resistance that are more relevant to the clinic. Such a model will also provide a powerful tool to identify those patients more likely to benefit from treatment with a therapeutic agent or combinations of agents.

The aim of the first part of my PhD was to develop a consistent, robust method for growing human colorectal tumours in *ex vivo* culture in our lab so that the therapeutic effect of AZD6244 and other agents could be studied in a range of human tumours.

Results:

3.1 – Development of an *ex vivo* model of human tumour explants

Prior to the start of my PhD the lab had developed a method for culturing explants of normal human intestinal mucosa, based on those described by Browning *et al* and Autrup *et al*. (*175-177*). The method was very successful and allowed normal mucosa to grow in culture for up to 16 days (Brady *et al*, Manuscript in

preparation). Therefore, I used this protocol as a starting point for developing a method to grow colonic tumours.

Briefly, colorectal tumours obtained at the time of resection were immediately placed in medium then transferred to the lab. Here they were washed to remove endemic colonic bacterial cultures, cut into 1-2mm^2 fragments then inserted in cuts made at the edges of a sterile stainless steel mesh. The mesh was balanced in the centre well of a culture dish containing supplemented Waymouth culturing media which had previously been determined by the group to provide optimal results for normal colonic mucosa explants. According to Browning *et al (175)* and Autrup *et al* (*176, 177*) the presence of 95% O_2 significantly increased oxygenation allowing for longer culturing periods. As this was shown to be the case with the normal mucosa samples cultured in the host lab, I also applied this condition to my tumour explants. Tumours were cultured in a chamber with 95% O_2 and 5% CO_2, and incubated at 37ºC with gentle rocking. Culturing media was replaced daily, and samples were maintained in culture for four days. Similar to normal mucosa explants, the objective was to maintain the cultures with constant oxygen supply, and intermittent access to humidity and nutrients.

I found that this method was inadequate to the culture of tumour tissue due to the nature of the specimens. Unlike normal mucosa, tumour tissue could not be stretched over the mesh and the process of trying to secure the tumour fragments in the mesh caused great damage to the tissue, often even disintegrating it. Also, tumour samples differ greatly in their consistency, and while soft, mucous rich fragments were relatively easy to insert in the mesh; solid, fibrous fragments could not be firmly attached without damage, and would eventually become free from the mesh

due to the rocking movements. I attempted to grow four tumours using this method, but quickly concluded that it was unsuitable due to these technical difficulties.

The stainless steel support provided to the normal mucosa samples was an inorganic material that was not likely to interact with the tissue. Regardless of this explants grew supported by their own microenvironment. I therefore hypothesised that tumour explants could also grow supported by their own stroma only. To test this hypothesis, I simply immersed the tumour fragments in supplemented Waymouth media under the same culturing conditions. Three tumours were cultured using this method. Samples were collected after 24h, 5 days, 10 days and 16 days in culture, paraffin embedded, then sectioned and H&E stained. Five fields of view were captured for each section (one section per sample) then the number of epithelial cells per field counted and the average calculated (see materials and methods for quantification details). The results obtained with the first sample cultured show a clear, time dependent reduction in the number of epithelial cells, with the most dramatic decrease being observed in the first 24h (Figure 3.1). Morphological analysis also showed continuous loss of tissue morphology through time in culture in all samples cultured using this method (Figure 3.1). These results suggested that tissue was being damaged by direct contact with forceps in the process of daily transfer to new culture dishes.

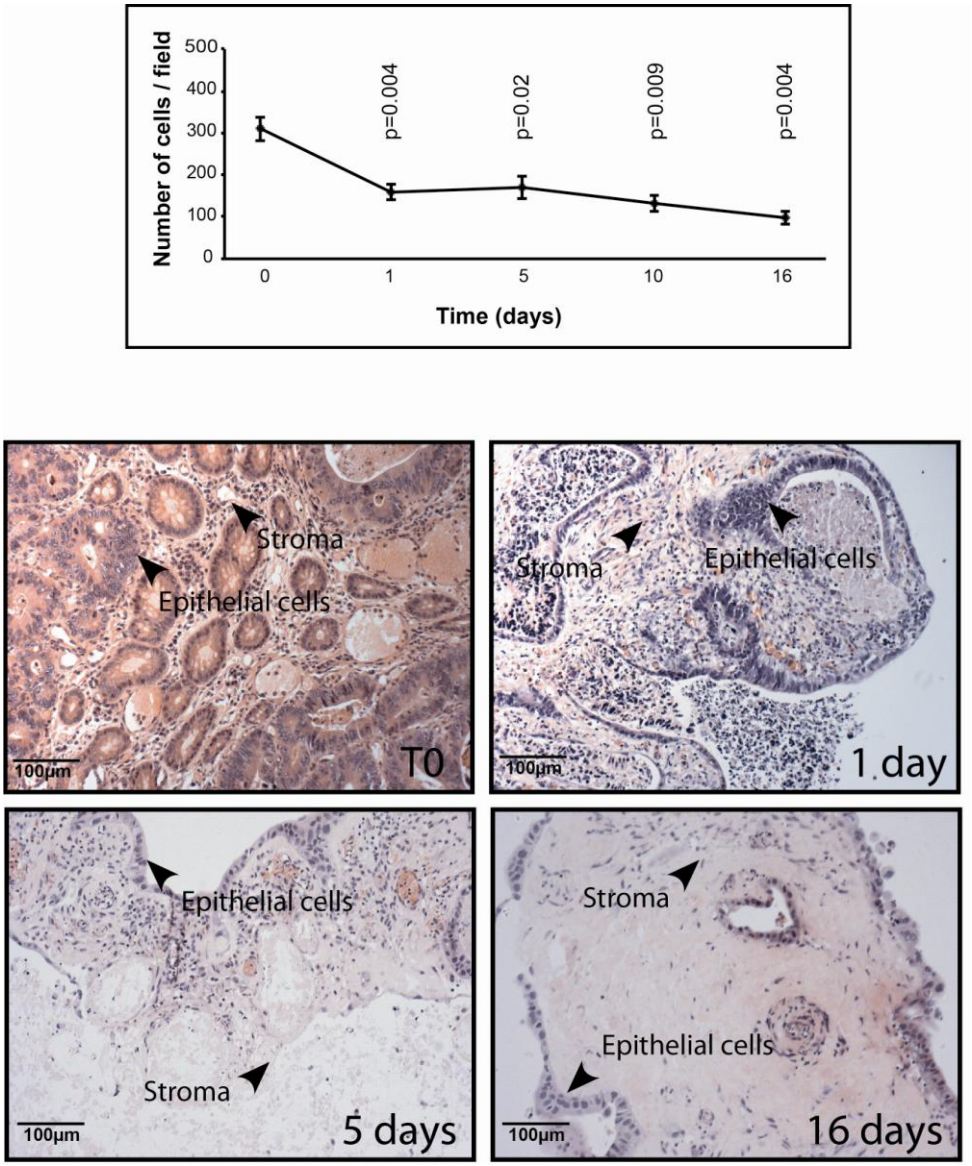


Figure 3.1: Tumour morphology is not maintained through time in culture due to dramatic decrease in the number of epithelial cells. H&E stained sections of a tumour cultured for up to 16 days using the floating method (bottom) show that original morphology was not maintained through time in culture. Loss of morphology resulted from the dramatic decrease in the total number of epithelial cells observed as early as 1 day in culture and thereafter, as shown in the graph (top). Data was obtained from 5 fields of view captured per time point. One tail Student's t test was used to calculate p values. Epithelial cells were identified according to their typical morphology as shown in H&E stained sections.

3.1.1 Evaluation of tissue support platforms: filter paper

One problem with the above method was that the daily transfer of tissue fragments into new medium caused mechanical damage to the tissue. Based on this evidence and on the results previously obtained by the group with normal mucosa samples, I hypothesised that the use of an inert tissue support platform would work as a raft for the daily transfers to new culture dishes, but also as a structure to which the tumours could attach to and grow.

Previous reports had suggested that lens or filter paper could be used as a low cost and effective support platform to allow the growth of normal and malignant tissues *ex vivo* (*179-181*). Hearn *et al* showed that embryonic intestinal tissue cultured on filter paper support was viable for up to 10 days with maintenance of morphology and differentiation patterns evidenced by the expression of typical markers (*179*), and Yarnell *et al* described a method of culturing tumour biopsies that included floating rafts of lens paper for tissue support and maintenance of the cultures in pressurised chambers (*181*). Therefore, I proceeded to culture the tumours under the conditions previously used, but on filter paper rafts so that daily transfer to a new culture dish could take place without mechanical damage, and so that the tumour explants could be maintained on a support. In total, ten tumours were evaluated for their ability to grow on filter paper disks. Each tumour was divided into a maximum number of 10 pieces (dependent on original size). The tumour fragments were then placed individually on filter paper disks and immersed in media in the centre well of the dish. I continued to use the supplemented Waymouth media that had been successful for growing normal mucosa. I also continued with the oxygenation and media replacement regime. I hoped that, using these growth

conditions and providing the tumours with a support would allow the re-growth observed in normal mucosa. Samples were harvested after 0, 1, 5, 10, 16, 20, 24 and 30 days of initial plating, fixed then paraffin embedded. The number of data points per tumour was dependent on the initial size of the sample.

To investigate culture effects on tumour growth and morphology, H&E stained sections were analysed and the number of cells epithelial cells per field of view was quantified as above. These data clearly demonstrated that there is a time dependent decline in the number of epithelial cells in the sample, and that this decline is most abrupt within the first 24h in culture. H&E stained sections also show considerable loss of tissue morphology especially at later data points (Figure 3.2).

To further analyse cell growth and death in culture, immunohistochemistry was performed on tissue sections using the proliferation marker Ki-67 and apoptosis marker active caspase-3. Interestingly, I found that even though there was a rapid decrease in the number of epithelial cells in the samples after 24h in culture, there was no change in the percentage of proliferative cells (Figure 3.3b). However, there was a significant increase in the percentage of cells positive for caspase-3 (Figure 3.3d). These data would suggest that upon plating on filter paper, a large number of epithelial cells undergo apoptosis, which results in cell loss, but the remaining cells continue to proliferate.

Since hypoxia has been shown to contribute to tumour growth (*182, 183*), one tumour was cultured in parallel under high and low oxygen conditions. Sample performance in culture was evaluated by H&E and immunohistochemical analysis as described above. Analysis of the number of epithelial cells at each time point, obtained from H&E stained slides, showed no difference between samples cultured

in the presence of high or low oxygen levels. Both groups displayed the same dramatic decrease in the number of epithelial cells after 24h in culture and thereafter (Figure 3.4). Analysis of proliferation marker Ki-67 also showed no difference between the two groups. In both sets, Ki-67 expression remained stable for 24 hours then dropped considerably after 5 days in culture (Figure 3.4).

In contrast to Ki-67, I found that, at least in this one sample, growth under hypoxic conditions dramatically increased the level of apoptosis, as indicated by increased number of cells staining positive for active caspase-3 after 1 day in culture (Figure 3.4). I concluded from these data that, even though the sample number was small, growth in hypoxic conditions is unlikely to reverse the initial cell death/loss observed when tumours are grown on filter paper.

From this set of data I concluded that the filter paper method, despite presenting a solution for the minimisation of damage caused by the daily changes to a new culturing dish, does not provide the requirements for tissue growth. The loss of typical morphology through time in culture indicates that the method does not contribute to the maintenance of tissue microenvironment leading to continuous tissue degeneration. Also, the high initial levels of apoptosis make it inadequate for drug testing as it would be difficult to ascertain whether apoptotic levels would be caused by the culturing method itself or as a result of treatment.

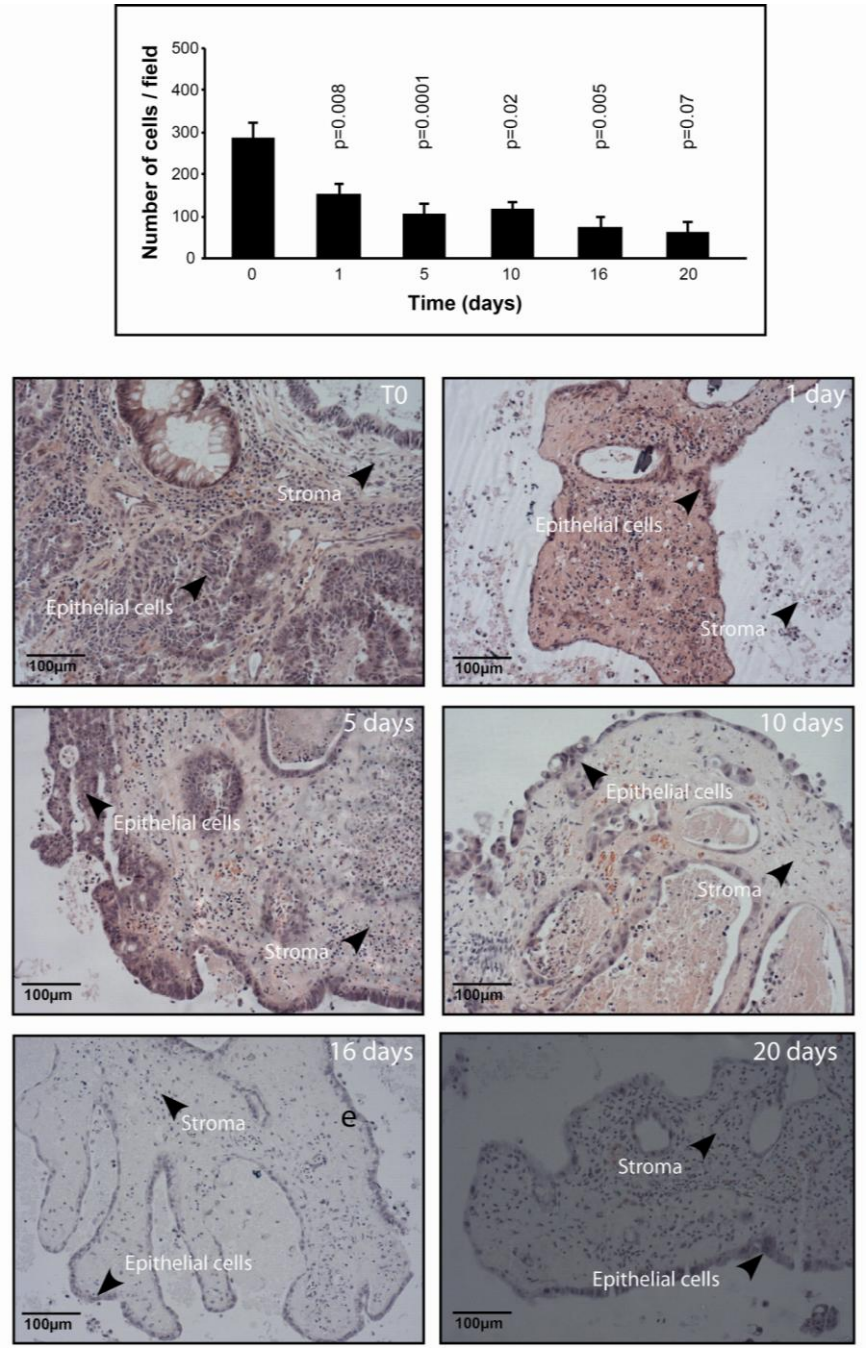


Figure 3.2: Histological analysis of tumours cultured with filter paper support. H&E stained sections show loss of original morphology in a tumour cultured on filter paper for up to 20 days (Bottom). Graph shows the decrease in the number of epithelial cells observed at 1 day in culture and thereafter in ten samples cultured using this method (Top). Images were captured from 5 independent fields of view per time point per tumour and the number of cells quantified. Epithelial cells were identified according to their typical morphology as shown in H&E stained sections. Data presented are the pooled results for all tumours (Average of T0, n=10; 24h, n=7; 5 days, n= 10; 10 days, n=5; 16 days, n=4; and 20 days, n=2 +/-SE). One tail Student's t test was used to calculate p values.

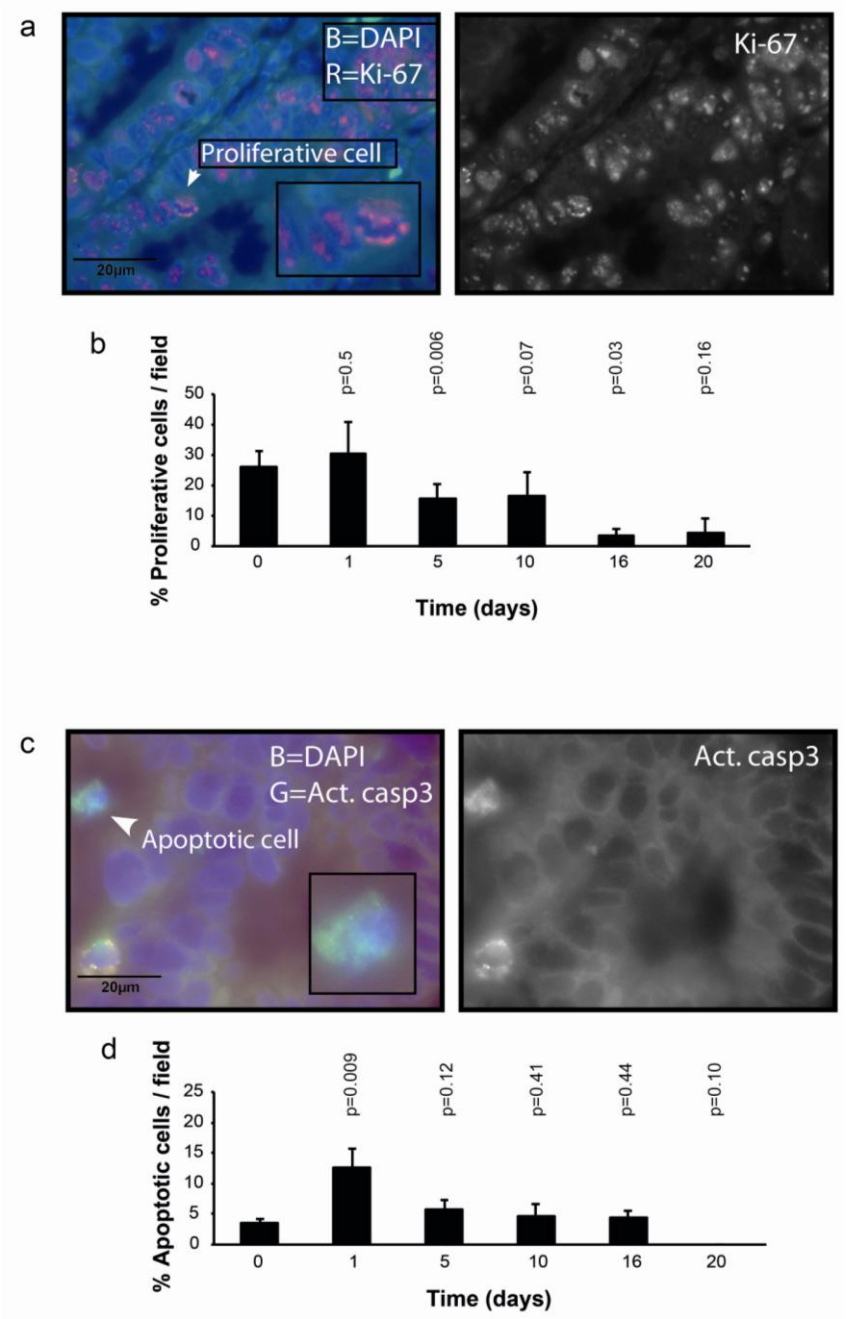


Fig. 3.3: Proliferation and apoptosis rates in samples cultured with filter paper support. Figures (a) and (c) show examples of sections stained for proliferation marker Ki-67 (a) and apoptosis marker active caspase-3 (c). Images were captured at a magnification of 1000x. Image (a) shows a field of view with a large number of Ki-67 positive cells which can be easily identified in the single channel image on the right. Image (c) shows a field of view with only one clearly positive active caspase-3 cell. Single channel image on the right confirmed positive staining. The percentage of positively stained cells for each marker was quantified from 5 fields of view per tumour per time point. Quantification results are shown in graphs (b) and (d). Data are the average for T0, n=10; 24h, n=7; 5 days, n=10; 10 days, n=6; 16 days, n=4; 20 days, n=2 +/-SE. One tail Student's t test was used to calculate p values.

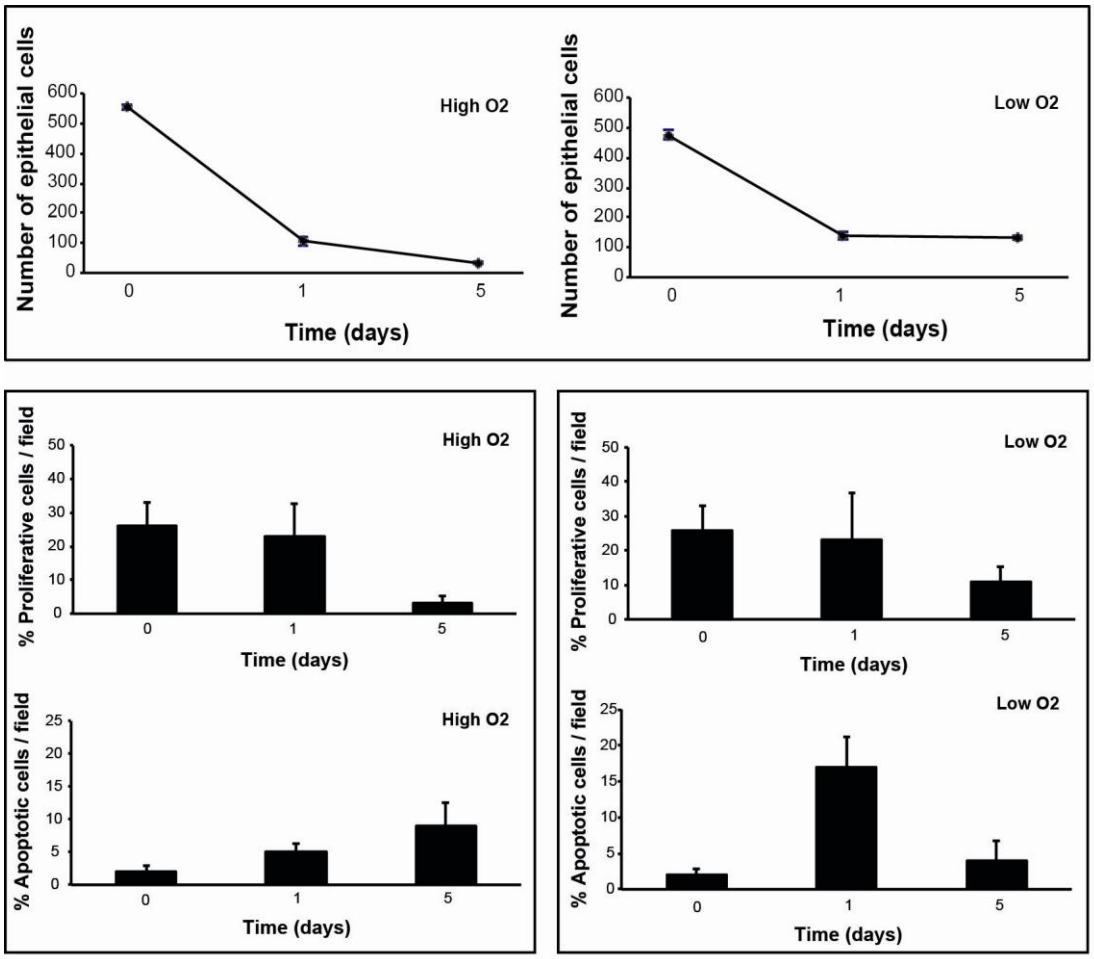


Figure 3.4: Lower O2 conditions did not improve the performance of a tumour cultured with filter paper support. Top graphs show histological analysis of the number of epithelial cells in one tumour sample cultured in parallel on filter paper with high and low oxygen supply. Decrease in the number of epithelial cells through time in culture has occurred in a similar fashion in both groups of samples. The same is observed for proliferation marker Ki-67. However, expression of apoptosis marker active caspase-3 seems to indicate that, contrary to what was expected, samples performed better under high oxygen conditions. Data was obtained from 5 fields of view per sample per time point. Data are the average of T0, n=2; 1 day, n=2; 5 days, n=2 +/- SE.

3.1.2 Evaluation of tissue support platforms: collagen gels

As the use of filter paper as a tissue support platform proved unsuccessful for the maintenance of tissue morphology and homeostasis, I hypothesised that a type of tissue support that better resembles the tumour microenvironment found *in vivo* would contribute to the maintenance of tissue integrity and allow for growth.

The importance of the extracellular matrix in cell growth, differentiation and survival has long been established. Though the nature of the relationship between cells and extracellular matrix varies with the type of cell, adhesion between cells and the extracellular matrix is fundamental for the establishment of signalling routes that determine cell behaviour (*184*). For example, in a variety of cell types, including intestinal epithelial cells, loss of adhesion to the extracellular matrix rapidly induces apoptosis (*185*). The extracellular matrix is mainly composed of collagen which accounts for 90% of its dry weight in most tissues, and of which type I is the most common (*186*).

The successful use of collagen gels in *ex vivo* culture systems of different types of tissue has been described by several authors (*168, 187-190*), including Vescio *et al* and Willson *et al* who, in two independent publications, reported the use of this tissue support platform in long term culture of colorectal tumour tissues obtained from patients (*169, 191*). Therefore, my next approach was to culture the tumour explants using type I collagen gels for tissue support. Briefly, 1-2mm^2 tumour fragments were placed individually on commercially obtained type I collagen gels previously set in 24-well plates, and covered with supplemented media. As the method used to culture normal mucosa had not been successful for tumours, I reverted to culturing the tissue using a method described in the literature in which

Waymouth media was replaced with MEM, and instead of incubation in a pressure chamber with controlled O_2 levels, tissues were incubated with 5% CO_2. Tumours were cultured at 37ºC as previously, but without rocking.

To evaluate tumour growth, ten samples were cultured using this method. H&E stained sections of tumour samples show that typical morphology is maintained for up to 12 days in culture (Figure 3.5), and immunostaining with epithelial cell marker KRT18 (*192*) shows that even though the number of epithelial cells drastically decreases in the first 24h in culture, it remains constant thereafter (Figure 3.5) suggesting that samples undergo a period of adjustment to the new environment after which they stabilise. This notion is also supported by the results obtained with proliferation marker Ki-67 and apoptosis marker active caspase-3 (Figure 3.6). The proliferative rates greatly decreased after 24h in culture but became constant after day 5, which coincides with the pattern observed with the epithelial cell marker KRT18. The increase in the number of apoptotic cells in the first 24h in culture followed by a drastic decrease at 5 days also reflects the number of epithelial cells during this period (Figure 3.6).

Histological and immunohistochemical data obtained with this set of tumours clearly demonstrated that the number of epithelial cells decreased through time in culture indicating that growth has not occurred. However, data also suggests that, after an initial period of catastrophe, tumours remain stable in culture for up to seven days.

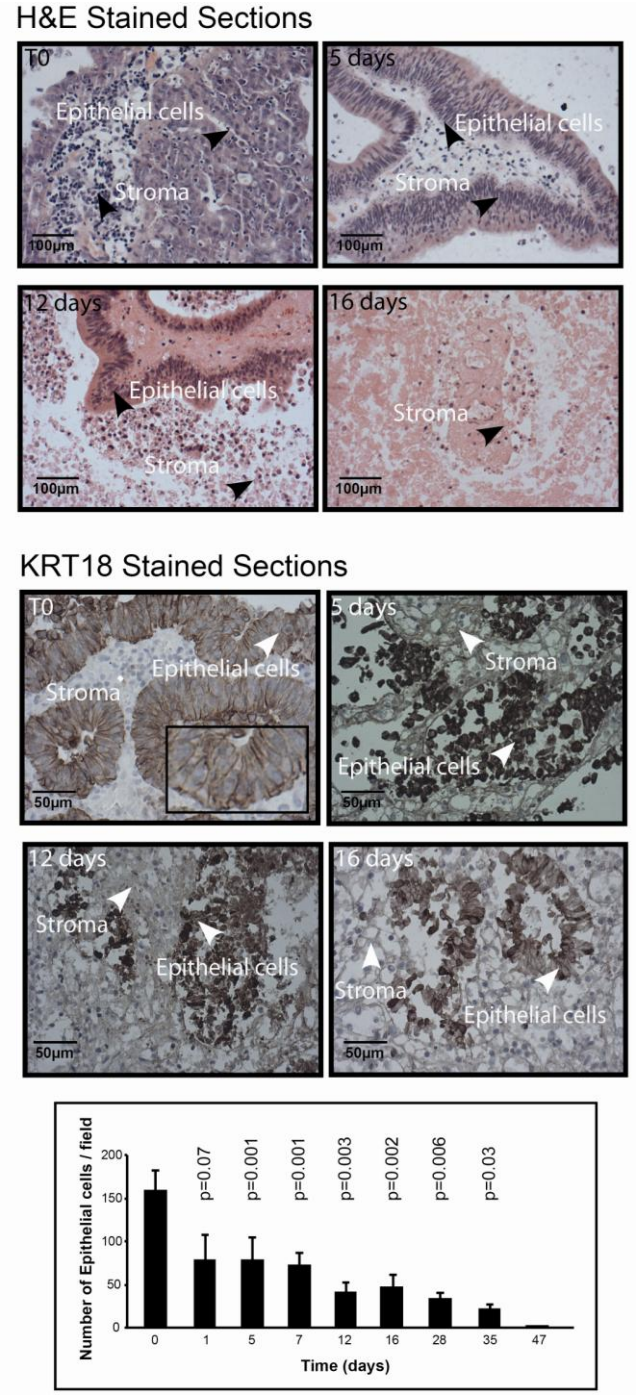


Figure 3.5: Histological analysis of tumours cultured using collagen gel support. Top figures show examples of H&E stained sections of the same tumour at indicated time points. Bottom figures show tissue sections of the same tumour at different time points stained for epithelial cell marker KRT18. Images were captured at 400x magnification. Graph shows the variation in the number of epithelial cells through time in culture. Five fields of view were captured per sample, per time point, and the number of KRT18 positive cells quantified. Data shown are the average of T0, n=6; 24h, n= 2; 5 days, n=5; 7 days, n=4; 12 days, n= 4; 16 days, n=4; 28 days, n=2; 35 days, n=2; and 47 days, n=1, +/-SE. One tail Student's t test was used to calculate p values.

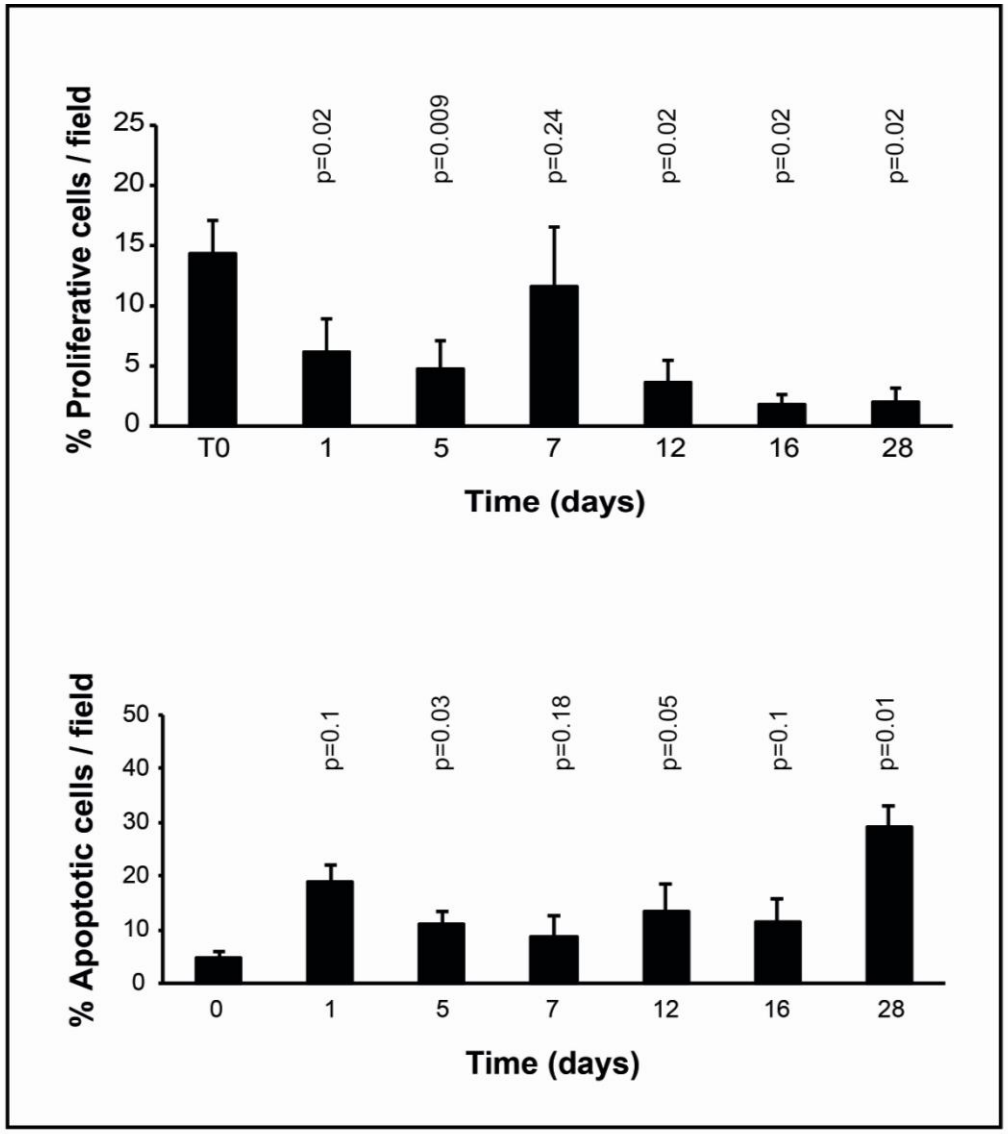


Figure 3.6: Proliferation and apoptosis in tumours cultured using collagen gel support. Top: graph shows the percentage of Ki-67 positive cells per field of view per time point. Bottom: graph shows the percentage of active caspase-3 positively stained cells per field of view through time in culture. For each marker five fields of view per time point per sample were analysed. Data presented here are the average of all tumours collected for each time point: T0, n= 6; 24h, n=2; 5 days, n=6; 7 days, n=5; 12 days, n=4; 16 days, n=4, and 28 days, n=3, +/- SE). One tail Student's t test was used to calculate p values. Data indicates that explants stabilise in culture after an initial period of adjustment to the culturing conditions.

I consistently observed the formation of cell outgrowths from the tumour when they were placed on collagen gel beds. This occurred in a time dependent manner and was evident from as early as 24h after plating in the vast majority of the explants. Generally, the number of cells invading the collagen gels increased in number over several days. An example is shown in Figure 3.7. Similar outgrowths of tumour cells had been observed within the institute when breast cancers were placed on collagen beds and it was believed that these outgrowths represented an example of invasive tumour. Furthermore, similar observations were previously reported by Freeman *et al* (*168*).

These data were particularly interesting as they suggested that although the main tumour body was not growing, cells were invading into the medium. I hypothesised that this cell invasion/growth may act as a good model for tumour growth. Therefore, I proceeded to characterise the nature of the outgrowths.

3.1.3 Characterisation of tumour outgrowths

Eight tumour samples were cultured on collagen gels as described above, with the objective of characterising the tumour outgrowths, and exploring the therapeutic potential of this model. Tumour fragments were plated onto collagen gels as above then each fragment was imaged prior to harvest and at 0, 1, 2, 5, 7, 16 and 28 days.

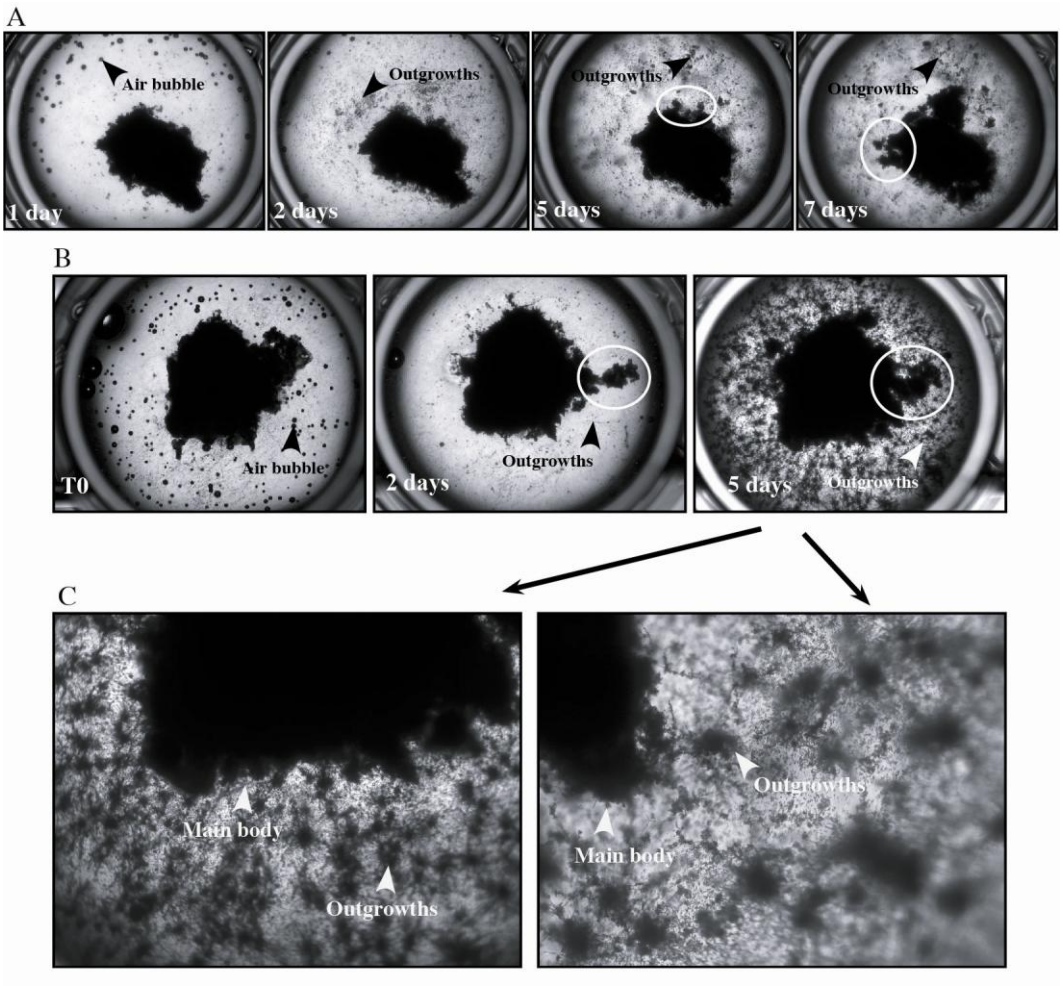


Figure 3.7: Formation of outgrowths observed in tumours cultured on collagen gels. The figure shows two examples of tumour samples grown on collagen gels (A and B). Images were captured at indicated times while still in culture (1x magnification). In A, formation of outgrowths was observed 2 days after plating with continuous increase in number and size through time in culture. By day 7, the main body of the tumour also appears to have grown. In B, clear outgrowth formation was only observed at day 5, but occurred in a much more dramatic fashion than in A. C shows zoomed in images of the outgrowths observed in B. These were clearly moving from the tumour onto the gel.

These data demonstrated that in all 8 tumour samples, cell aggregates grew from the tumour into the collagen matrix in at least one fragment cultured. In 75% of tumours all fragments cultured displayed this phenomenon. In total, 165 tumour fragments were placed in culture for this series of experiments and I found that cells grew out the tumour to visually form growths in the matrix in 148 of these fragments. This represents 89.6% of all tumour fragments. However, the degree of outgrowth formation varied from tumour to tumour, but also within the same tumour. Qualitative analysis of the density and size of the outgrowths revealed different patterns of growth on to the gels. The outgrowths were then graded into four different categories according to these patterns and the number of wells displaying each grade was quantified. Wells that displayed a small number of outgrowths of reduced size scattered onto the gels were classified as grade 1. Wells that presented a large number of small outgrowths and few larger ones scattered onto the gel were classified as grade 2. Wells that presented large outgrowths occupying ~75% of the gel area were classified as grade 3, and wells that presented very large outgrowths that fully covered the whole area of the gel were classified as grade 4. Figure 3.8 shows examples of different grades observed, and how frequently they occurred.

To investigate the origin and nature of these structures, I initially tried to fix and embed the cell outgrowths and the collagen gels. However, this proved difficult due to the small size of the cell bodies and the fragility of the collagen gels. Therefore, I proceeded to digest the collagen gels with collagenase IV to fully release the cell bodies from the gels. I then extracted protein so that outgrowths could be analysed using western blot analysis.

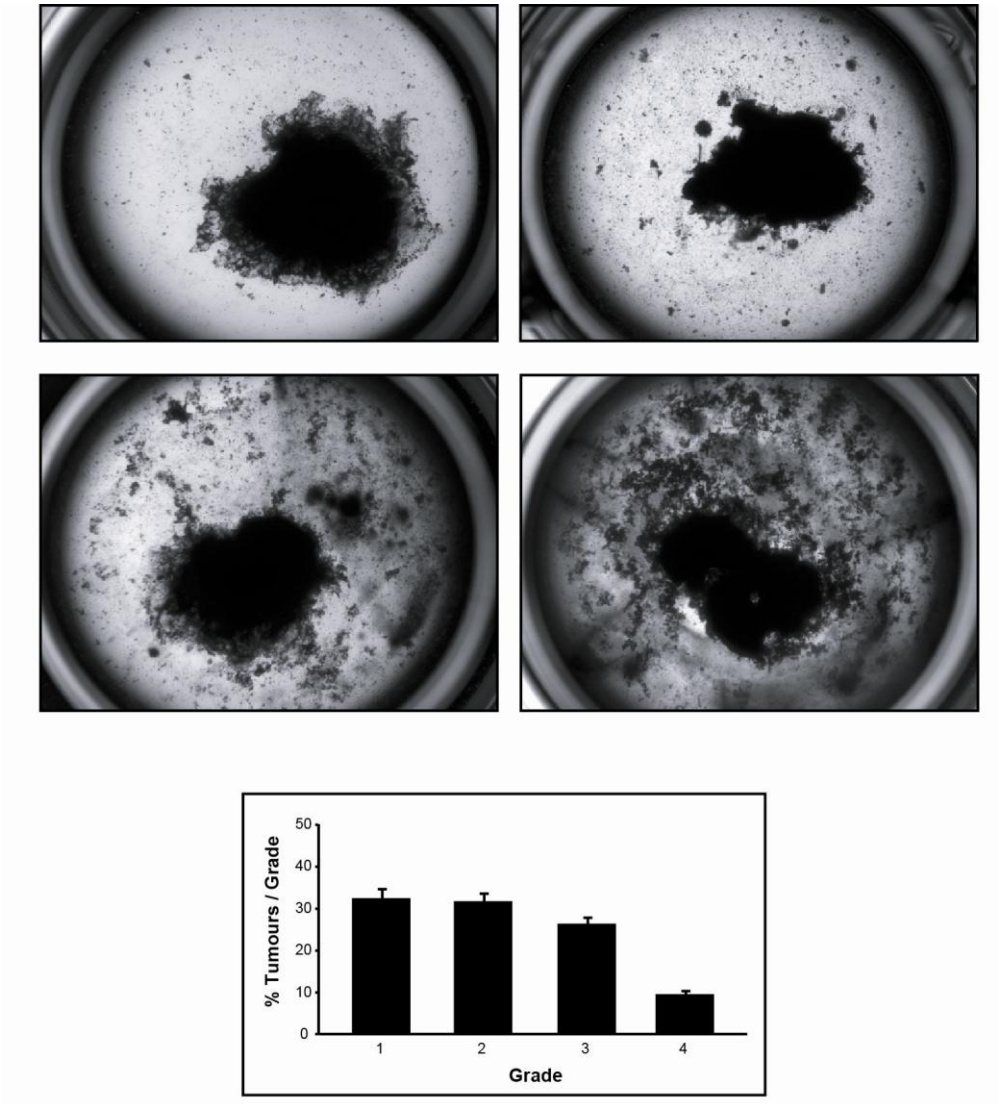


Figure 3.8: Grade of outgrowth formation and frequency. Top – figure shows examples of each grade of outgrowth formation observed. Images were obtained from different wells at 7 days in culture using a Nikon AZ100 macroscope at 1x magnification. Qualitative analysis of the density and size of the outgrowths revealed distinct patterns of growth on to the collagen gels. The number of wells displaying each grade was counted. The graph (bottom) shows the frequency at which each grade was observed.

At the point of harvest, the main body of the tumour was also removed from the collagen gels and immediately frozen for protein extraction and western blot analysis. To determine whether the observed tumour outgrowths were of epithelial nature, I performed western blot analysis with antibodies to EpCam (Epithelial cell adhesion molecule). This is a type I membrane glycoprotein exclusively expressed in epithelial tissues and epithelia derived neoplasms. It is involved in a variety of processes such as signalling, cell migration, proliferation and differentiation (*193, 194*). EpCam expression is greatly increased in rapidly proliferating tumours but reduced in normal tissues (*195*). The protein can occur in 4 distinct forms (34-40KDa) as a result of differential glycosylation. The glycosylation pattern of EpCam is reported to be tumour specific (*196, 197*).

Firstly, I found that in all cases, EpCam was detected in protein extracted from tumour outgrowths, suggesting that epithelial cells were present in this population. When analysing the pattern of EpCam expression, I found that there were multiple EpCam bands at T0 but after 24h in culture only one rather intense band of smaller size was detected, indicating that the glycosylation status of the protein had changed as a result of the culturing conditions. At later data points, another larger band of ~60kDa was observed in some samples, suggesting further changes to the protein. Importantly, these patterns of EpCam expression were observed in both tumour bodies and outgrown cells, suggesting the two populations had a common origin (Figure 3.9).

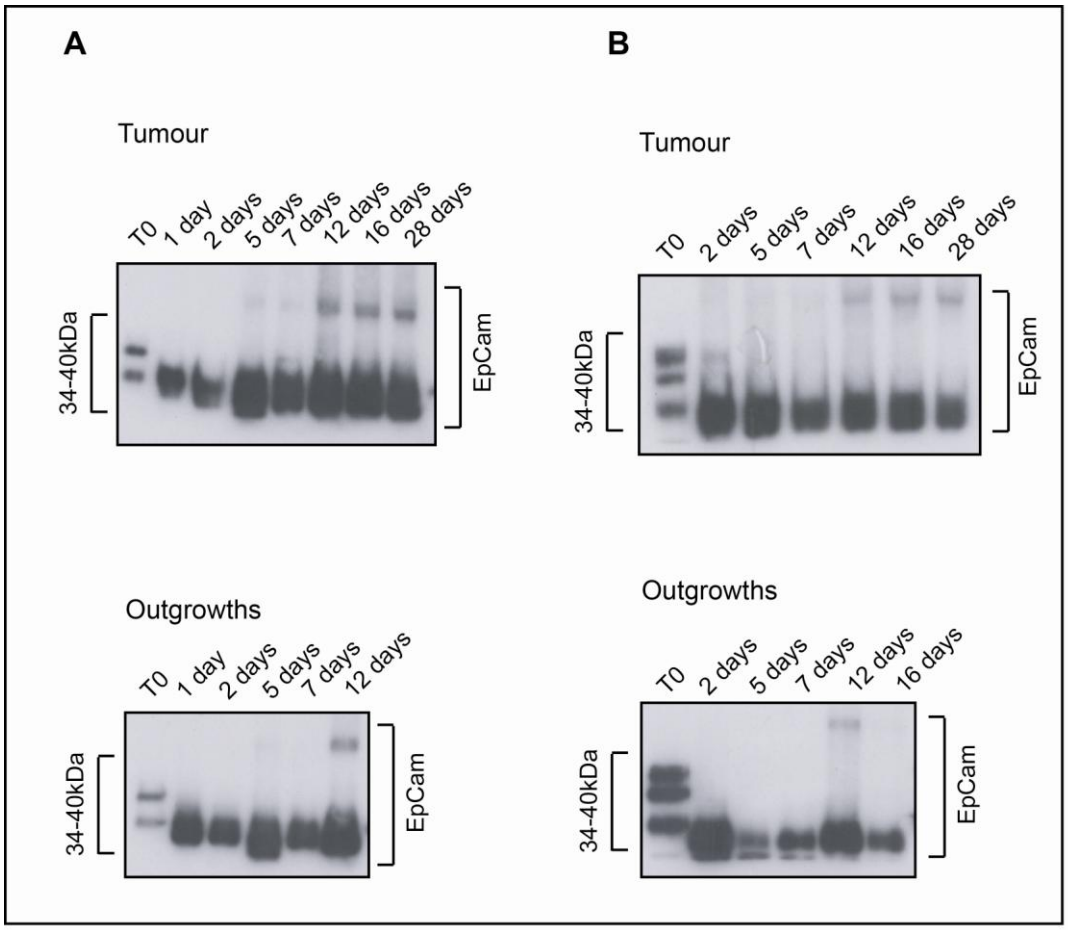


Figure 3.9: Pattern of expression of EpCam suggests that cell outgrowths are of epithelial origin and derived from the main body of the tumour. Figure shows western blot analysis of epithelial cell marker EpCam in main tumour body and cell outgrowths of two tumour samples (A and B). Western blot analysis was used to compare the levels, and post-translational status of the epithelial cell marker EpCam in the main body of the tumour versus the outgrowths. Figure demonstrates that the marker is detectable in outgrown cells, and that the post-translational status in these structures parallels that of the main tumour body. T0 sample is the same in tumour body and outgrowth gels.

To confirm these data, western blot analysis was performed using the epithelial cell marker, KRT18. In contrast to the results obtained with EpCam, I found that KRT18 could not be detected in outgrown cell samples, while this was readily detectable in samples from tumour bodies (Table 3.1). Given that KRT18 expression is reported to be higher in differentiated epithelial cells (*198*) while expression of EpCam is inversely correlated with differentiation (*199*) the discrepancy may suggest that the outgrown epithelial cells are less differentiated and more aggressive than tumour epithelial cells.

Based on the EpCam results, I concluded that the tumour outgrowths were epithelial in nature and were behaving like the epithelial cells in the main tumour body once in culture. Next, I wished to determine whether the outgrowing cells were viable. In order to do this I performed MTT assays. MTT reagent, a tetrazolium salt, is incorporated and reduced to formazan by live cells only, causing a change in colour of the reagent (*200*). MTT reagent was added to the culturing media and cultures were incubated for 4h at 37ºC. Changes in colour were recorded with macroscopic imaging. Results show that, in four tumours analysed, outgrowths remain viable in culture up to at least 1 day (Figure 3.10).

Based on the consistency with which tumour outgrowths were observed, my data suggesting that they were of epithelial nature and the data showing they were viable, I concluded that, this model had potential for testing therapeutic agents.

Sample	KRT18		EpCam	
77T	**Tumour**	**Outgrowths**	**Tumour**	**Outgrowths**
T0	+	n/a	Not performed	
5 days	+	+	Not performed	
78T				
T0	+	n/a	+	n/a
2 days	+	+	+	+
5 days	+	-	+	+
7 days	-	-	+	+
12 days	-	-	+	+
16 days	-	-	+	+
79T				
T0	+	n/a	+	n/a
1 day	+	+	+	+
2 days	+	+	+	+
5 days	+	-	+	+
7 days	+	-	+	+
12 days	+	-	+	+
16 days	+		+	
28 days	+		+	
80T				
T0	+	n/a	+	n/a
1 day	+		+	
2 days	+	+	+	+
5 days	+		+	+
7 days	+		+	+
12 days	+		+	+
16 days	+		+	
28 days	+		+	
81T A				
T0	+	n/a	+	n/a
2 days	+	-	+	+
5 days	+	-	+	+
7 days	+	-	+	+
12 days	+	-	+	+
16 days	+	-	+	+
28 days	+		+	

Table 3.1: Expression of epithelial cell markers KRT18 and EpCam. Table shows the summary of the results obtained with western blot analysis for epithelial cell markers KRT18 and EpCam in four tumour samples at different time points in culture. (+) indicates the presence of bands while (-) indicates the absence of bands of each marker. While EpCam expression is similar in tumour and outgrowths samples, KRT18 is barely present in the latter.

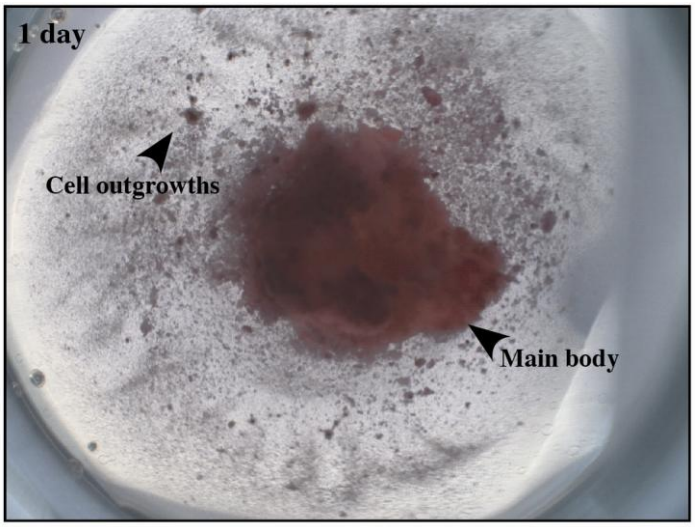


Figure 3.10: Cell outgrowths from the main tumour are viable after 1 day in culture. MTT assay performed on a tumour sample cultured on collagen gels after formation of outgrowths. The pink colour of the MTT reagent indicates that outgrown cells are viable.

3.1.4 Collagen gel support: the potential for testing therapeutic drugs

My previous data suggested that this model may be suitable for testing the response of patient tumour explants to different therapeutic agents. To test this suggestion, I firstly utilised aspirin as a model anti-tumour agent. The group in which my PhD was undertaken have been working on the mechanisms by which aspirin acts against colon cancer cells for a number of years (*133, 201*). Therefore, the timing of aspirin events and a number of specific markers of aspirin response are known. Furthermore, this agent is known to inhibit proliferation and mediate apoptosis of a wide range of colorectal cancer cells *in vitro* (*133*), acting independently of the mutational status of the cancer (*202*).

I hypothesised that agents that inhibit tumour growth may inhibit proliferation/mediate apoptosis of outgrowing cells, thus blocking outgrowth formation. In order to test this hypothesis, five tumour explants were plated in collagen gels as above. At the point at which the outgrowths were apparent (24-48h), cultures were treated with aspirin (0-10mM) for 4-6 days. Following harvest, western blot analysis was used to examine specific markers of aspirin response in the main body of the tumours and in outgrown cells, as above. However, the data obtained from this series of experiments were inconclusive. I found that it was technically very challenging to perform western blot analysis on the outgrown cells (before and after treatment) as cell numbers were small giving very low protein yields. I also found that the markers of aspirin response analysed, Cyclin D1 and IκB, were not expressed in all tumours (data not shown). While this was interesting, it did not allow for aspirin response to be determined.

Due to the difficulties in working with outgrown cells, I next tested aspirin effects on the main tumour bodies using an alternative protocol. I hypothesised that if tumours were treated immediately after plating, increased apoptotic rates as a result of treatment could be measured, and typical markers of response would still be expressed at detectable levels. Therefore, I proceeded to treat tumour samples at time 0 (initial plating) for 24h only. Eight tumours were treated using this method.

Immunohistochemical analysis with antibodies to active caspase-3 demonstrated that, as had been previously observed (see 3.1.2), the tumours underwent catastrophic apoptosis within the period of the experiment (compare T0 and 0uM in Figure 3.11a). Furthermore, treatment regimes did not significantly increase these rates. A lack of a significant dose responsive effect of the agent on cell proliferation and apoptosis can be seen in individual tumours (data not shown) and if data from all tumours is combined (Figure 3.11a). Western blot analysis demonstrated that aspirin, which is an acetylating agent, induced a dose-dependent increase in total protein acetylation, confirming penetration of the drug into the tumour (Figure 3.11b). Decreased levels of Cyclin D1 is an early marker of aspirin response (see Introduction) (*135*). However, there was minimal change in the levels this protein (Figure 3.11c). In keeping with my immunohistochemistry results, western blot analysis indicated that aspirin had little effect on levels of proliferation or apoptosis, as indicated by the markers PCNA and pro-caspase-3 respectively (Figure 3.11d).

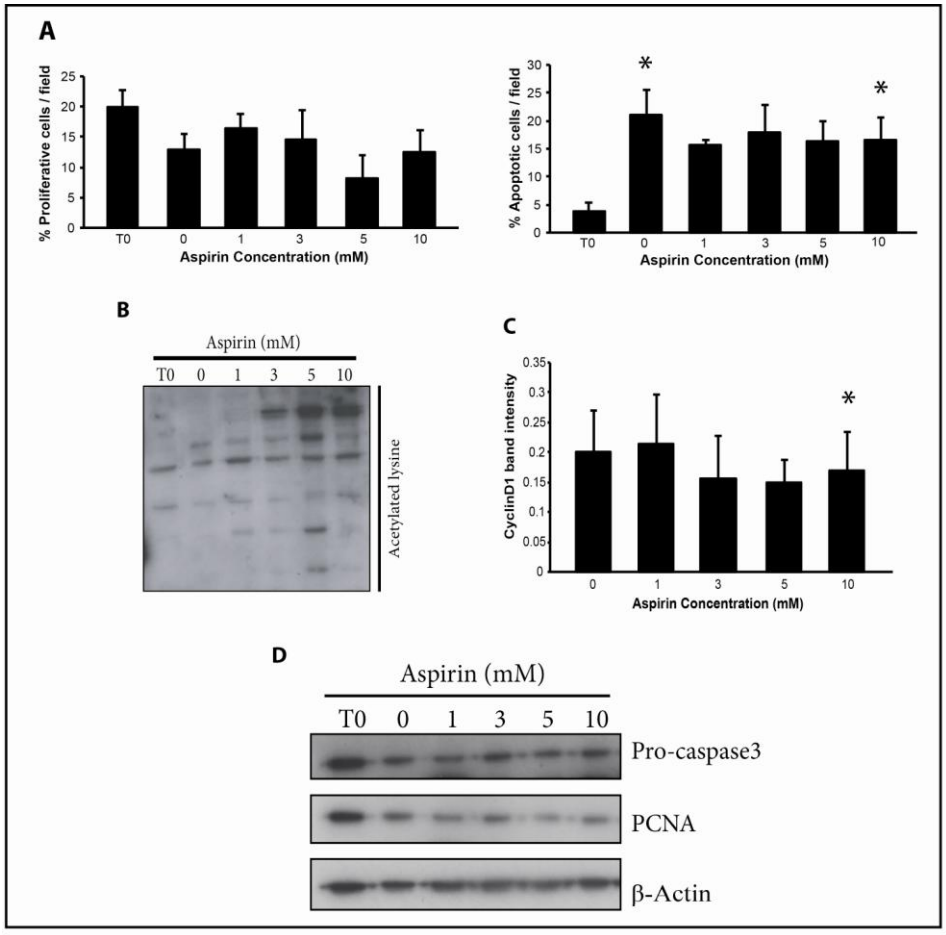


Figure 3.11: Markers of response to aspirin treatment. (A) Immunohistochemistry for Ki-67 and active caspase-3 used to examine the effect of the agent on proliferation and apoptosis, respectively, in tumour samples treated for 24h with aspirin in dose response studies. Five fields of view were captured per sample per concentration, and the average number of positive cells for each marker was quantified. Data shown are the pooled results for five tumours +/- SE. One tail Student's t test was used to calculate p values. An asterisk (*) above the data point indicates p≤0.05. (B) Western blot analysis showing protein acetylation as a result of aspirin treatment in tumours. Protein acetylation is clear in samples treated with 3-10mM aspirin. (C) Western blot analysis was used to examine aspirin effects on known marker of response, Cyclin D1. Image J analysis used to quantify intensity of bands on western blots indicated no significant change in Cyclin D1 levels in response to aspirin. Data shown are the pooled data for five tumours for each dose. One tail Student's t test was used to calculate p values. An asterisk (*) above the data point indicates p≤0.05. (D) Western blot analysis of apoptosis marker Pro-caspase-3 and proliferation marker PCNA. Expression of both markers remained constant in response to aspirin.

To determine whether this lack of response was due to the agent, or a failing of the model, I next tested Oxaliplatin. This agent is routinely used in the clinic as a therapy for colorectal cancer and has previously been shown to induce cell death in colorectal cancer cell lines (*203*). Oxaliplatin induces cell cycle arrest at the G1-S phase of the cell cycle caused by a decrease in expression of Cyclin B and CDK1 (*204*). Three tumours were treated with 0-10μM of the agent. Western blot analysis indicated that the agent induced minimal change to the levels of these markers in all 3 tumours analysed. In keeping with a lack of response to the agent there were minimal changes to levels of PCNA and pro-caspase 3 (Figure 3.12).

Taken together, these data would suggest that this method of tumour growth is not appropriate for therapy testing without substantial further optimisation.

3.1.5 – Culturing of human colorectal tumours: spheroid cultures

The use of multicellular tumour spheroids in cancer research has been reported in the literature for nearly 40 years. Multicellular tumour spheroids obtained from human cancer cell lines are used as an alternative to monolayer cultures to study the regulation of tumour cell physiology and therapeutic responses in a 3D context (*205, 206*). Multicellular spheroid cultures can also be obtained from normal intestinal mucosa from mice (*207, 208*), or from human normal intestinal epithelia (*163, 187*). These systems enable the study of proliferation, migration and differentiation patterns of normal intestinal mucosa.

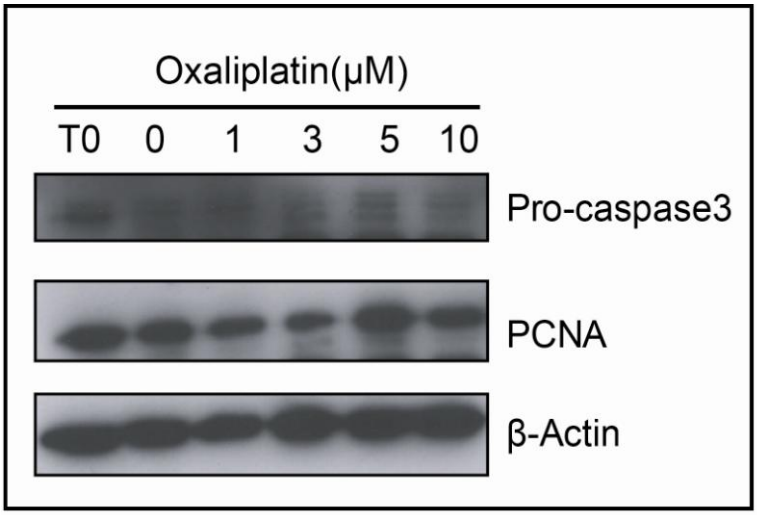


Figure 3.12: Markers of response to Oxaliplatin treatment. Western Blot analysis of apoptosis marker Pro-caspase-3 and proliferation marker PCNA in samples treated with increasing concentrations of oxaliplatin for 24h. Data shows a lack of dose responsive effects of oxaliplatin.

Multicellular tumour spheroids obtained from disaggregated human colorectal tumours have also been reported, and despite some controversy regarding the spheroid ability to reproduce the tumour's original *in vivo* environment and maintain it's individual characteristics, these systems have shown potential as models to study different aspects of tumour biology and response to therapeutic agents (*161*, *162*). Therefore, I next used the protocol recently described by Kondo *et al*. (*161*) to grow spheroids from human tumour samples.

Briefly, patient tumours obtained at the time of resection were immediately transferred to the lab, washed and minced. Minced tissue was then washed by centrifugation and re-suspended in supplemented MEM media containing collagenase IV. Tissue was incubated at 37ºC for 2h with vigorous rocking. After digestion with collagenase IV, samples were filtered through a stainless steel mesh with a pore size of approximately 500μm and then passed through a 40μm cell strainer to remove smaller fragments, washed by centrifugation, re-suspended in supplemented stem cell media and cultured at 37ºC with 5% CO_2. Fresh media was added to the cultures every other day. Spheroid formation was monitored using a confocal microscope.

Six tumour samples were cultured using this method and consistent formation of spheroids of different sizes (Figure 3.13) was observed in all samples following 24-48h in culture. However, four of the samples cultured were discarded after 48h due to the presence of contamination. Expansion of the spheroid cultures to 24-well plates containing previously set type I collagen gels was attempted with one of the tumours cultured.

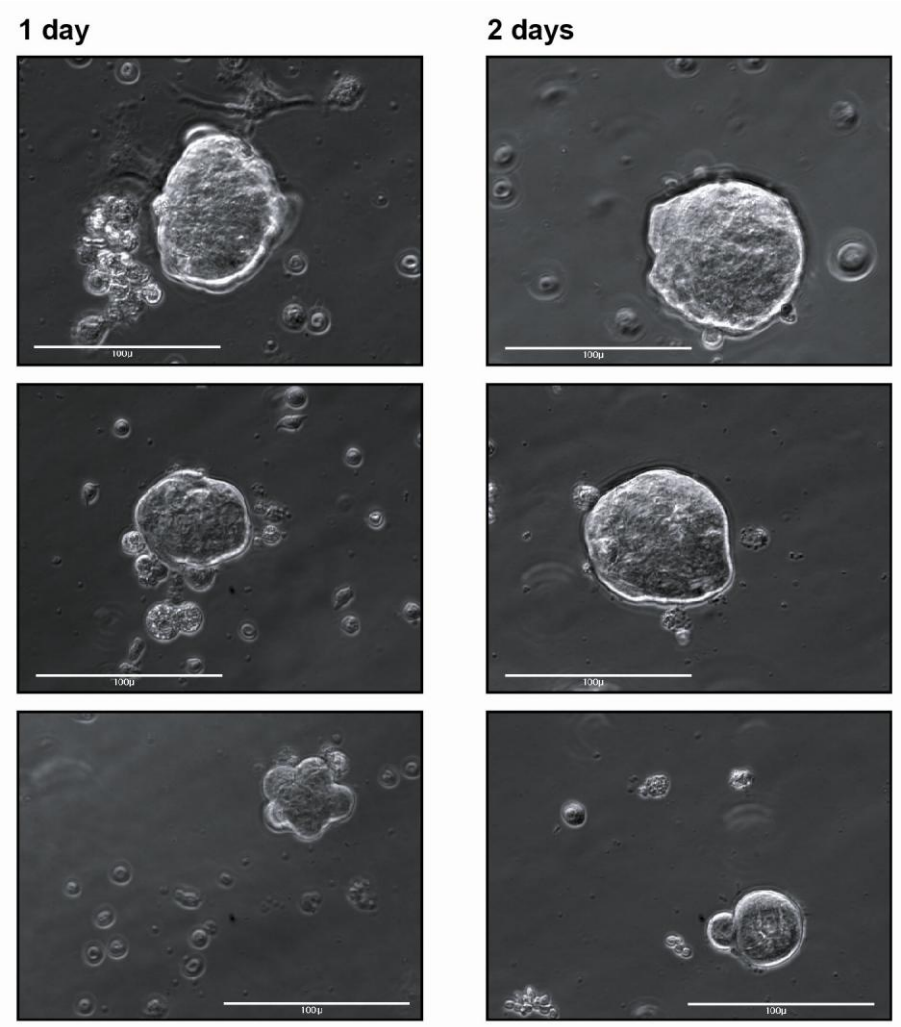


Figure 3.13: Multicellular tumour spheroids. Images show tumour spheroids obtained from disaggregated fresh colorectal tumour tissue and cultured in supplemented stem cell media for 1 (left) and 2 (right) days.

However, re-setting into collagen gels appeared to stop the growth of the spheroids. Instead I found that simply splitting the cells to new culturing dishes containing supplemented MEM was enough to stimulate the formation of new spheroids, and the growth of existing ones. One of the tumours was expanded in culture for several weeks using this method. Surprisingly, I observed that in culture dishes that did not have the medium replenished the spheroid number was increased. This was possibly due to the increased proximity between spheroids or increased concentration of signalling molecules in the media. It may also be that lack of nutrients and oxygen supply stimulated tumour growth. Splitting of these cultures into fresh media did not result in further growth, though spheroids appeared viable during the whole period in culture.

Despite the fact that this method appeared extremely promising, I did not proceed to test its potential in drug response assays. Further use of the method would require characterisation of the spheroids to ascertain if the tumour's individual initial features were maintained and for how long, and the development of new methods of analysis. At this point in my PhD, I could not afford to pursue a method that may ultimately not be suitable, as the previous methods had not been.

Discussion:

The development of an *ex vivo* model of human colorectal tumours proved to be extremely challenging and time consuming. The initial culturing method, adapted from the protocol used to grow normal intestinal mucosa, and the floating method, showed that normal and malignant tissues have different growth requirements. The

floating method also indicated that colorectal tumour cultures require a support platform with which the tissue can interact with and grow.

The use of lens or filter paper as a support platform for tissue growth was previously described in the literature. Yarnell *et al* (*181*) described a method with which tumour biopsies were cultured on lens paper support floating over culturing media supplemented with antibiotics, amino acids, glucose and 10% calf serum, and incubated in a sealed chamber containing 5% CO_2. The group were able to maintain cultures of myxofibrosarcoma of the thigh for 48h, and use the model to study response to therapeutic agents, methotrexate and melphalan. Similarly, Hearn *et al* (*179*) reported the long term growth of embryonic intestinal tissue from mice on filter paper support platforms immersed in media supplemented with antibiotics, amino acids and 10% foetal calf serum, and incubation with 5% CO_2 with media changes every two days. Based on these reports, I hypothesised that colonic tumours could be grown on filter paper rafts for extended periods of time, but even though I used culturing conditions very similar to those described by these authors, tissue growth was not observed in any of the tumours cultured. This could be due to the daily media changes, which continuously depleted the cultures from signalling molecules; and the rocking movements which prevented the tissue from attaching to the filter paper. These conditions were not used by Yarnell *et al* or Hearn *et al*, but were maintained in my cultures based on the results previously obtained in the lab with normal mucosa samples.

Interestingly, Yarnell *et al* were only able to maintain tumours in culture for 48h which is not far from the results obtained with my panel of tumours grown on filter paper support, and might explain why the group did not continue to use the

method and later on moved on to cultures on agar gels (*209*). The method described by Hearn *et al* was successfully used in other publications and applied to the study of several aspects of gastrointestinal biology (*210, 211*). These reports indicate that the filter paper method is appropriate to the culture of normal intestinal epithelia but not ideal for the culture of malignant tissues.

My next approach was to grow the tumours on collagen gel beds in order to provide a support platform that better resembled the tumours *in vivo* environment. I followed a protocol described by Freeman *et al* (*168*) in which tumours were dissected and placed on previously hydrated collagen gels then covered with MEM media supplemented with antibiotics, amino acids, glutamine and 10% foetal calf serum. The method allowed for the growth of tumours of various origins including lung, colon and breast carcinomas for up to 28 days, during which the formation of outgrowths from the main tumour body on to the gels was observed. Similarly, the formation of outgrowths from the main tumour on to the gels was also observed in my panel of tumours grown on collagen gels. This indicated that cells were either growing or migrating from the main tumour body. As tumours are comprised of both epithelial and stromal cells, outgrowths could have originated from either source. The outgrown cells showed strong expression of EpCam which would not be detected in cells of stromal origin. Furthermore, the glycosylation status of this EpCam resembled that of EpCam in the original tumour. Therefore, I concluded that the cells were of epithelial origin. Unfortunately, low protein concentrations, and difficulties in fixing and embedding the cell outgrowths limited further assessment of the nature of these structures. Ideally, I would have used markers such as Vimentin

(a type III intermediate filament that can be used to identify fibroblasts (*212, 213*)) to determine if a stromal cell population was also present in the outgrowing cells.

Another question that remains unanswered is whether outgrown cells were growing out of the main tumour body or migrating, the latter could in part explain the decline in the number of epithelial cells in the main tumour. However, assessing for the expression of invasion markers like Laminin 5 (*214*) or matrix metalloproteases (*215*), and Snail and E-cadherin which are involved in the epithelial to mesenchymal transition (*216*) was also limited by the factors mentioned above. The use of a cytospin system and subsequent immunostaining with these markers was also proposed, but this was limited by the fact that outgrowths originated from patient tissue and had to be handled in a Control Level 2 laboratory. I also attempted to use tracking dyes, and live imaging of the cultures but these assays also presented a few technical limitations.

Vescio *et al* (*169*) previously demonstrated that tumour tissue grown on collagen gels could be used to investigate the response to a variety of therapeutic agents, amongst which cisplatin and 5-flurouracil. The group investigated therapeutic effects in 20 major classes of tumours, including colon, and found that sensitivity patterns of various tumour types were similar to those found *in vivo*. Based on this and on the observations made with my panel of tumours cultured on collagen gel beds, I proceeded to test the potential of this model in therapeutic response assays. However, I found that I could not detect a pattern of response to treatment with aspirin or oxaliplatin in my panel of tumours which indicated that the use of this model required further optimisation.

One optimisation procedure that could have been tested was the use of tissue slices instead of tumour fragments, as described by Vaira *et al* (*167*). This method would allow for better oxygenation and access to nutrients which could have stimulated explant growth. However, the culture of tumour slices was limited by the lack of access to a microtome capable of sectioning fresh human tumour samples. The use of other types of tissue support like Gelfoam sponge as described by Pirnia *et al (217)* was also considered. However, the use of Gelfoam sponge required a medical prescription. Maintaining the cultures in media supplemented with growth factors would be likely to increase proliferation rates strongly contributing to the tissue regeneration that I aimed for, however the cost of these supplements was an important limiting factor. One cheaper and readily available option that could have also been tested was the use of DMSO as described by Autrup *et al* (*176, 177*). The presence of DMSO could have increased access to nutrients by facilitating diffusion, and it could also have contributed to tissue preservation. The use of DMSO might have also facilitated the absorption of aspirin and oxaliplatin improving response to treatment with these therapeutic agents. In summary, various culturing conditions could have been attempted, but ultimately, time and continuous access to patient material which would have allowed for such conditions to be tested, presented the strongest limiting factors.

The results obtained with multicellular tumours spheroids suggested that this protocol could provide a reproducible and robust method for culturing colorectal tumours. Multicellular spheroids were obtained from all tumours cultured; and for one tumour, spheroids continued to form and grow after a number of passages, and

were maintained for several weeks. Thus, the use of this system would allow a much wider window for drug testing and other applications. Interestingly, observations made on this sample suggested that tumour tissue, or at least multicellular tumour spheroids would grow better under nutrient starvation and possibly hypoxic conditions, factors that are also present in the *in vivo* tumour environment. Proximity between the spheroids might also have contributed for the success of these cultures.

Despite being a promising method, culturing the tumours as multicellular spheroids also required further optimisation of the analysis methods so that full characterisation of the spheroids could be performed prior to testing the response to therapy. Morphological analysis to confirm the maintenance of tumours original features would have to be performed, and though it is possible to fix and embed the spheroids, the protocols in place would have to be adjusted to the small size of these structures. Once the maintenance of the tumour's individual characteristics was established, response to therapy could be assessed by immunohistochemistry, western blot analysis, and by assessment of spheroid growth in the presence and absence of drug. As proceeding with these optimisation assays could take several weeks or even months, the use of this protocol was halted.

In summary, 47 patient tumour samples were cultured using different methods based in the literature and adapted to the conditions in our laboratory (Table 3.2). Due to the limited success in growing colorectal tumour explants and the requirement for further optimisation of the methods used, I opted to use acute treatments in order to test the response to therapeutic agents in patient samples.

Method	Reference	Number of tumours	Time in culture (days)	Outcome
Stainless steel mesh / Floating	Used in out lab for culturing normal mucosa samples. Based on Browining et al (1969) and Autrup et al (1980)	5	4	Tissue damage due to daily changes into new plates with fresh media.
Filter Paper	Hearn et al (1999)	13	30	High apoptosis rates observed at 24h and thereafter. Lack of tissue regeneration.
Type I collagen gels	Freeman et al (1986) Vescio et al (1987)	23	47	Consistent formation of outgrowths from the tumour onto to the gels. High apoptotic rates observed in the main tumour bodies. Lack of response to treatment with growth inhibitor agents.
Multicellular tumour spheroids	Kondo et al (2011)	6	several weeks	Spheroid formation consistently observed after 24h in culture. Method is technically challenging and time consuming. Required culture Characterisation to be used in drug response assays.

Table 3.2: Summary of the methods used in the development of an *ex vivo* model of human colorectal explants. Table shows a list of the methods used in the development an ex vivo model of colorectal lesions, the literature reference for each of the methods, number of tumours cultured using each method, time that samples were in culture, and the outcome of the protocols used.

Chapter 4: Studying response to treatment with MEK1/2 inhibitor AZD6244

Introduction:

As outlined in the main introduction, mutations in KRAS and BRAF members of the MAPK pathway occur in more than 50% of colorectal cancers (*98*). These mutations are mutually exclusive and lead to constitutive activation of the MAPK pathway promoting tumour proliferation and survival (*96, 100*). Targeting the pathway downstream of KRAS and BRAF is therefore a promising therapeutic option.

AZD6244 is a highly selective allosteric and non ATP competitive MEK1/2 inhibitor with reported nanomolar activity against purified enzyme (*112, 113*). AZD6244 has completed Phase I and II clinical trials in a variety of tumour types, and it has been suggested for Phase III trials in *Ras*-mutated non-small cell lung carcinoma (*218*). With a Maximum Tolerated Dose of 100mg bid (twice daily) this agent has relatively low toxicity with rash and fatigue being the most commonly observed side effects. Treatment with AZD6244 fully inhibits ERK1/2 phosphorylation in lymphocytes from 12-*O*-tetradecanoylphorbol-13-acetate-treated whole blood used as a surrogate for tumour tissue within one hour of treatment (*115, 118*). However, disease stabilisation is generally reported as the best outcome, and criteria to identify patients that might benefit from treatment is still to be determined (*115-118*).

The mechanisms of response to AZD6244 have been extensively studied in cell lines and xenograft models. Studies developed in colorectal cancer cell lines show that treatment with AZD6244 can induce dose-dependent inhibition of ERK1/2 phosphorylation and consequent cell cycle arrest at G1 as evidenced by a decrease in Cyclin D1/CDK4 expression, and an increase of cell cycle inhibitors p21 and p27

(*95*). These studies also report a wide range of sensitivity to the anti-proliferative effects of the drug with some cell lines responding to doses of less than 100nM, while in others doses of over 10μM are needed to induce a response (*94*, *114*). *KRAS* and *BRAF* mutational status is thought to be an indicator of sensitivity to AZD6244. Data reported by Davies *et al* (*94*) demonstrated that cells carrying *KRAS* or *BRAF* mutations are more sensitive to the drug, presenting more growth inhibition in response to treatment than those with wild type genes. However, Balmanno *et al* (*114*) have demonstrated that neither *KRAS* or *BRAF* mutational status nor the degree of p-ERK1/2 inhibition observed after exposure to the drug, are good indicators of sensitivity to AZD6244. In this study, groups of cells harbouring the same mutation included cells that displayed high sensitivity, but also resistance to the drug. Similarly, cells presenting the same degree of p-ERK1/2 decrease displayed opposite outcomes in terms of growth inhibition, and caspase-dependent apoptosis occurred after 18h of treatment in sensitive cell lines, while in resistant cell lines this was not observed even after 72h.

While cell line studies are a good starting point for identifying markers of sensitivity, these do not recapitulate the complex signalling pathways and cell heterogeneity found in human tumours. Treatment of tumours growing in an *ex vivo* model is an ideal way to identify markers of sensitivity / resistance to this agent in a more realistic setting. My previous data suggested that regardless of growth conditions, tumours undergo extensive apoptosis within 24h of being placed in culture. Therefore, I set out to establish if response to AZD6244 could be measured using acute (short time point) treatments. I wished to use this method to determine

patient tumours sensitivity to AZD6244, and to identify markers of sensitivity / resistance that are more relevant to the clinic.

Results:

4.1 – MEK1/2 inhibition can be measured within 1h of treatment with AZD6244 in colorectal cancer cell lines

Markers of resistance / sensitivity to AZD6244 have been previously proposed in studies developed with colorectal cancer cell lines. However, the relevance of these data to human tumours is currently unknown.

I first used a panel of colorectal cancer cell lines in order to establish the minimum time point at which effects of AZD6244 could be measured. Based on data reported in the literature, I selected the colorectal cancer cells lines HRT18, HCT116 and RKO as these vary in their *KRAS* and *BRAF* mutational status and their reported sensitivity to AZD6244. RKO (*BRAF* mutant) and HRT18 (*KRAS* mutant) are intrinsically resistant to MEK1/2 inhibition by AZD6244 while HCT116 cells (*KRAS* mutant) are more sensitive (*114*). The concentrations of AZD6244 utilised were based on those that are clinically relevant.

Colorectal cancer cells were treated with 0, 0.1 or 3μM AZD6244 for 1, 3 and 6h then western blot analysis was used to examine effects on the ERK1/2 pathway. These data indicated that even at these relatively low concentrations, the agent inhibited ERK1/2 phosphorylation within 1h of treatment. For RKO and HRT18 cells, this effect was most apparent at the 3μM concentration. However, for the more sensitive HCT116 cells, 0.1μM of the agent was sufficient to completely inhibit p-ERK1/2 at this time point (Figure 4.1).

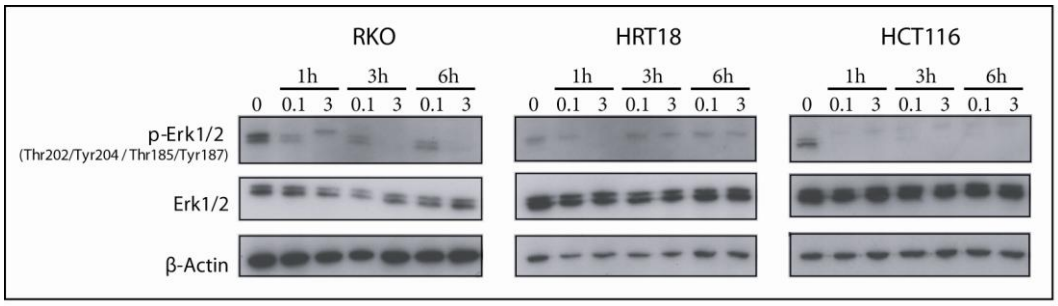


Figure 4.1: Response to AZD6244 treatment can be measured with acute (short) time points in colon cancer cell lines. Figure shows western blot analysis of p-ERK1/2 in a panel of cell lines. Inhibition of ERK1/2 phosphorylation is measurable after 1h of treatment with AZD6244 and its dose-dependent, especially in RKO and HRT18 cells. Native ERK1/2 levels are shown. β-actin was used as a loading control.

Interestingly, AZD6244 did not appear to inhibit p-ERK1/2 activity at the longer time points in HRT18 cells. This might be indicative of the feedback mechanisms described by Little *et al* (*92*) (see Introduction).

To further investigate whether sensitivity to AZD6244 can be measured using short term treatment with the agent, I next analysed the effects of the above treatment regime on cell proliferation and apoptosis. I found that within this time scale, the drug had a minimal effect on cell proliferation in all three cell lines tested, as indicated by immunocytochemistry for Ki-67 expression (data not shown). I also found that the agent had a minimal effect on apoptosis in HRT18 cells and RKO cells (Figure 4.2). However, dose-dependent induction of apoptosis was observed in the more sensitive HCT116 cells 1h after exposure to 0.1 and 3μM of the agent (Figure 4.2).

These data indicate that MEK inhibition by AZD6244 can be robustly measured after only 1h of treatment with either 0.1 or 3μM of drug, depending on sensitivity to the agent. It also shows that more sensitive colorectal cancer cell lines show growth inhibition effects at acute time points. These data suggested that differential sensitivity to AZD6244 may be identified using acute treatments with clinically relevant doses of the agent.

Based on the shortfalls of long term growth of tumours in culture and my findings with colorectal cancer cell lines, I proceeded to treat fresh colorectal tumour explants with AZD6244 in short term experiments. With these studies I aimed to: first, determine if acute treatments with AZD6244 can be used to identify sensitive and resistant patient tumours; and second, to identify markers of sensitivity to AZD6244.

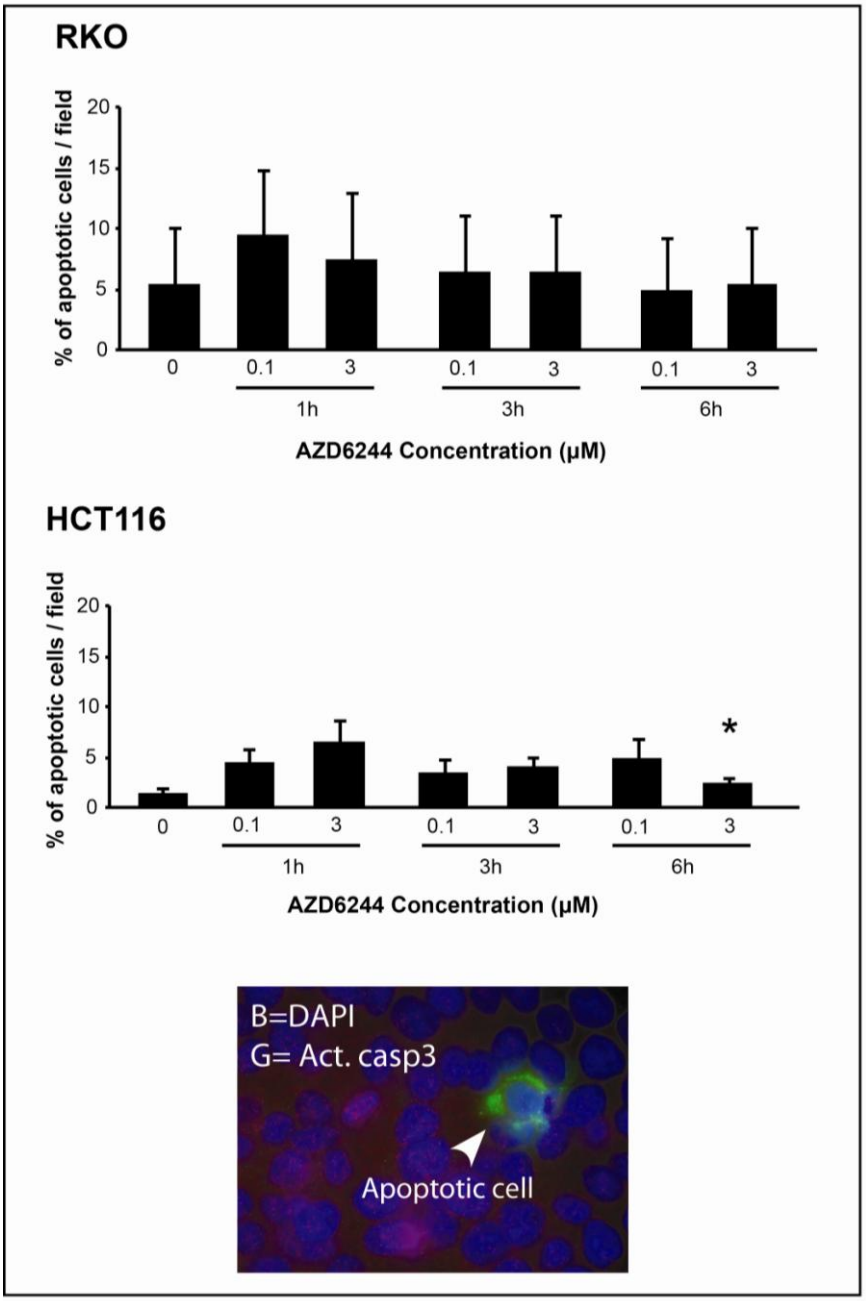


Figure 4.2: Induction of apoptosis can be detected after 1h treatment with AZD6244 in sensitive colon cancer cell lines. Immunocytochemistry with antibodies for active caspase-3 was performed to investigate the effects of AZD6244 on apoptosis. Ten fields of view were captured per sample per treatment then the percentage of positive cells quantified. Graphs show the average of 3 experiments +/- SE. One tail Student's t test was used to calculate p values. An asterisk (*) above the data point indicates p≤0.05. A 2-fold increase in apoptosis was observed in HCT116 cells (bottom) after 1h of treatment with this agent. In RKO cells AZD6244 had minimal effects on apoptotic rates (top). Image at the bottom shows an example of an apoptotic cell identified in HCT116 treated with AZD6244. Images were captured at 650x magnification.

4.2 – Establishment of a minimum time point at which AZD6244 effects can be measured in patient tumours

The protocol used in this study is outlined in Figure 4.3. Briefly, colorectal tumours samples collected at the time of resection were dissected into the relevant number of fragments, each of 1-2mm^2 in size. Each fragment was immersed in supplemented MEM media containing 0, 0.1 or 3μM AZD6244, and incubated at 37ºC with 95% CO_2. All samples had a DMSO concentration of 0.03%. A proportion of the tumour was retained for a T0 control. Two wells were set up for each treatment condition at each time point so that one piece could be frozen for protein extraction, and one piece fixed and embedded for immunohistochemical analysis. I was unable to routinely set up duplicate wells for each assay type due to the extremely small nature of the tumours.

The first three tumours were treated for 1, 3 and 6hrs. Using western blot analysis, I found that AZD6244 inhibited ERK1/2 phosphorylation in a dose-dependent manner. Furthermore, for all samples, this inhibition was evident within 1hr of treatment and showed no further decrease after 3 or 6hrs (Figure 4.4). Based on these data, and the fact that tumour samples were limited in size, I proceeded to treat tumours with 0, 0.1 or 3μM AZD6244 for 1h only.

In total, 23 patient tumours were treated using this method. The group of patients included 15 males and 8 females diagnosed with colorectal cancer between the ages of 39 and 80, with 78% of the patients being diagnosed between the ages of 55 and 80. Classification according to Duke's staging system revealed that one tumour was stage A, five were stage B and ten were classified as stage C. Pathology information of the remaining 4 patients was not available.

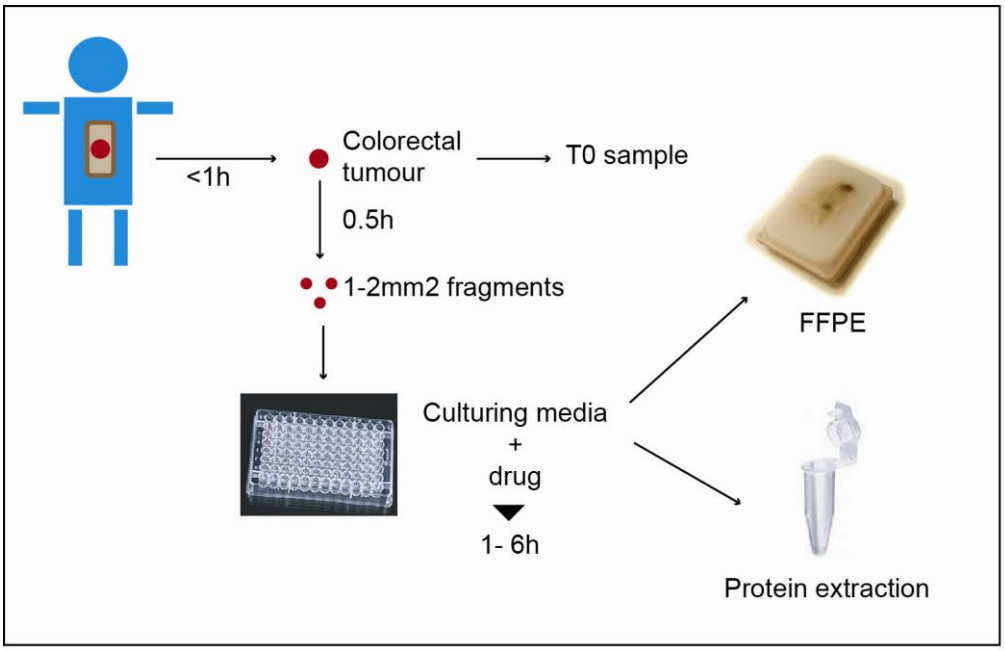


Figure 4.3: Schematic representation of the method. Tumours collected at the time of resection were immediately transferred to the lab, dissected and immersed in culturing media containing 0, 0.1 and 3μM AZD6244. Samples were harvested after 1-6h incubation and immediately frozen for protein expression, or fixed for immunohistochemical analysis.

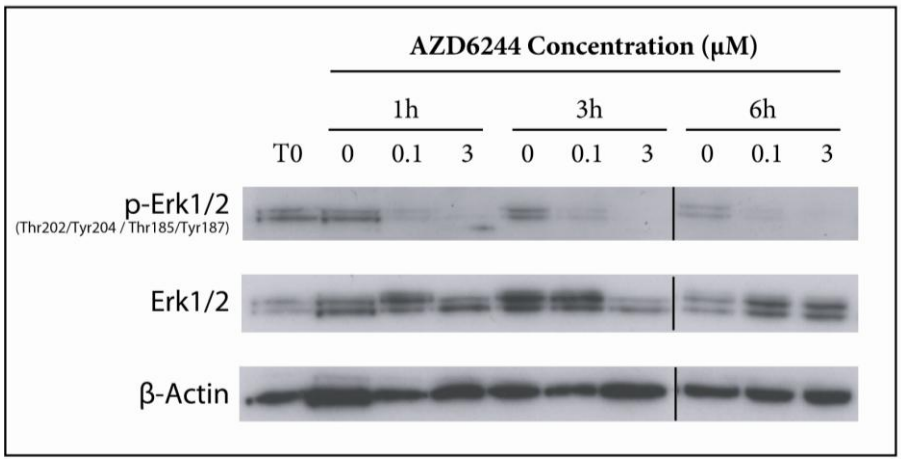
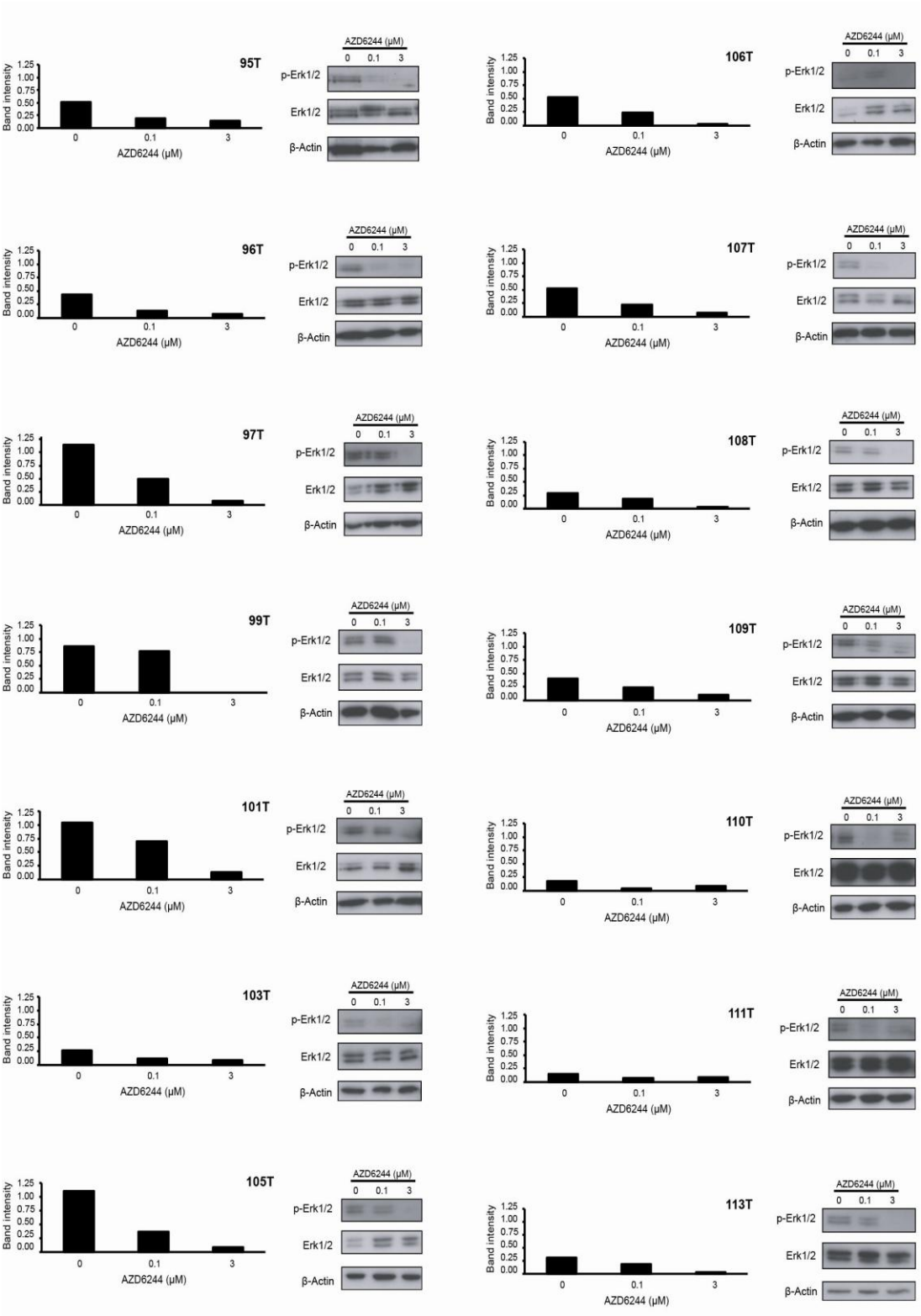


Figure 4.4: Inhibition of ERK1/2 phosphorylation in patient samples within 1-6h of treatment with AZD6244. Tumour explants were treated with AZD6244 for 1-6h and western blot analysis was performed on whole cell lysates with antibodies to native and phosphorylated ERK1/2. β-actin was used as a loading control. Data shows that inhibition of p-ERK1/2 is not time dependent, and AZD6244 effect can be clearly measured within 1h of treatment.

4.3 – Acute treatment with AZD6244 is sufficient to induce a pharmacodynamic response in human colorectal tumour explants

Studies developed in cancer cell lines and xenografts, and data from clinical trials show that treatment with AZD6244 rapidly inhibits ERK1/2 phosphorylation (*115*, *219*). Therefore, my first approach was to determine if p-ERK1/2 was inhibited in tumours treated with AZD6244 for 1h. I extracted protein from tumour samples frozen immediately after harvest and performed western blot analysis with antibodies for phosphorylated and native ERK1/2. Blots were quantified using ImageJ software. Native and phosphorylated ERK1/2 intensities were normalised using β-actin to correct for variability in protein loading. Phosphorylated ERK1/2 intensity was then normalised for total (native) ERK1/2.

Results show that AZD6244 inhibited ERK1/2 phosphorylation in all samples after 1h of treatment (Figure 4.5), and that in 21 samples treated with the agent, p-ERK1/2 inhibition occurred in a dose-dependent manner.


95T
106T
96T
107T
97T
108T
99T
109T
101T
110T
103T
111T
105T
113T
Band intensity
AZD6244 (μM)
0
0.1
3
p-Erk1/2
Erk1/2
β-Actin

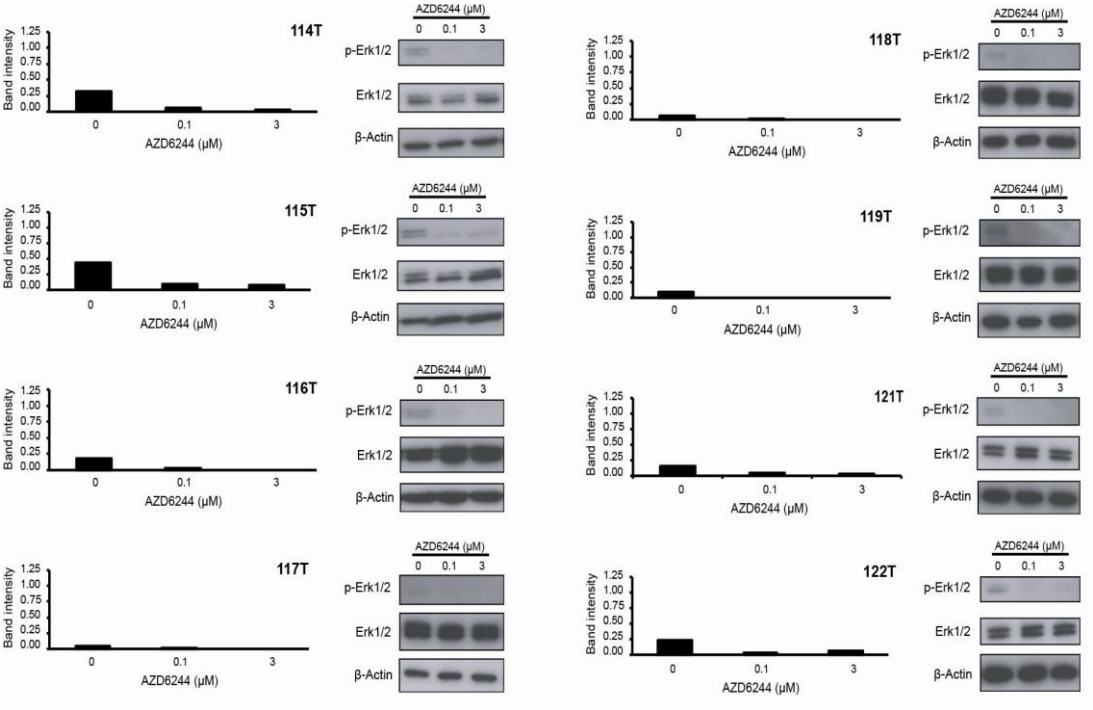


Figure 4.5: p-ERK1/2 inhibition in patient tumours treated with AZD6244. Tumours were treated with 0, 0.1 and 3μM AZD6244 for 1h and western blot analysis was performed on whole cell lysates with antibodies to native and phosphorylated ERK1/2. β-actin was used as a loading control. ImageJ was used to quantify blot intensities. Native and phosphorylated ERK1/2 levels were normalised with β-actin, and p-ERK1/2 was normalised with native protein. Western blots are shown on the right and respective quantification data are shown in graphs on the left.

Having demonstrated that this model was sufficient to detect p-ERK1/2 inhibition, I next wished to determine if this pharmacodynamic response translated into inhibition of proliferation and/or induction of apoptosis. Tumour samples were fixed immediately after harvest, embedded, then consecutive 4μm sections were used for immunohistochemical analysis for proliferation marker Ki-67 and apoptosis marker active caspase-3. Expression of these markers was analysed in T0 and all cultured samples. By comparing the T0 sample with the non treated control I was able to assess maintenance of tissue integrity in culture. To evaluate drug response, I compared samples treated with 0.1μM and 3μM AZD6244 with non treated (0μM) controls. I performed quantitative image analysis on 5 fields of view per marker per sample, captured at 1000x magnification. Imaging was done in a "blind" fashion to remove bias (see Materials and Methods). For each marker, the percentage of positive cells per field was counted then the average of the five fields determined. An example of these data, for one patient, is given in Figure 4.6. The full data set for all patients is presented in Figure 4.7. I found that for all samples and for both markers, the percentage of positive cells was consistent between the five fields of view (see error bars on individual graphs in Figure 4.7) giving strength to the data.

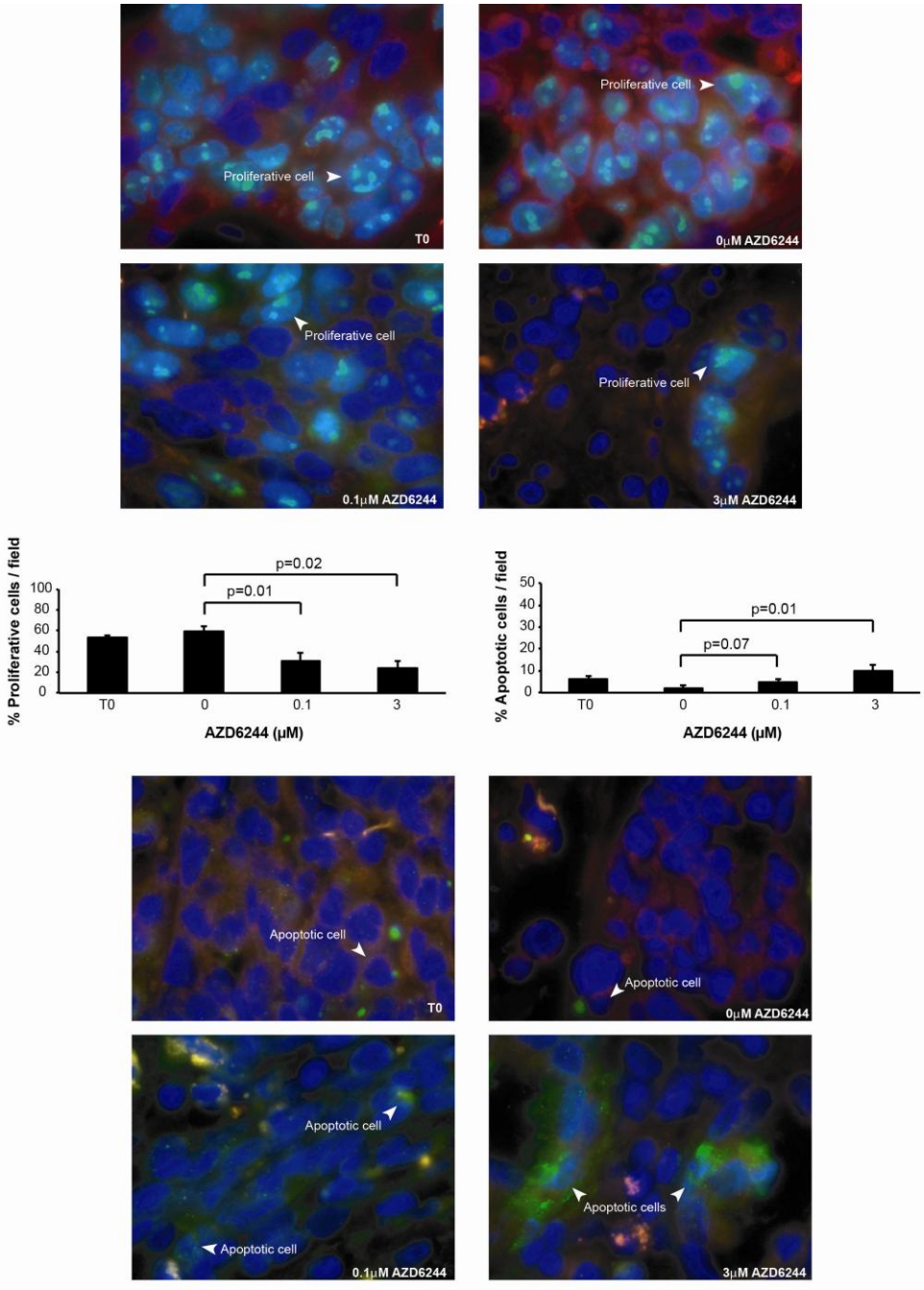
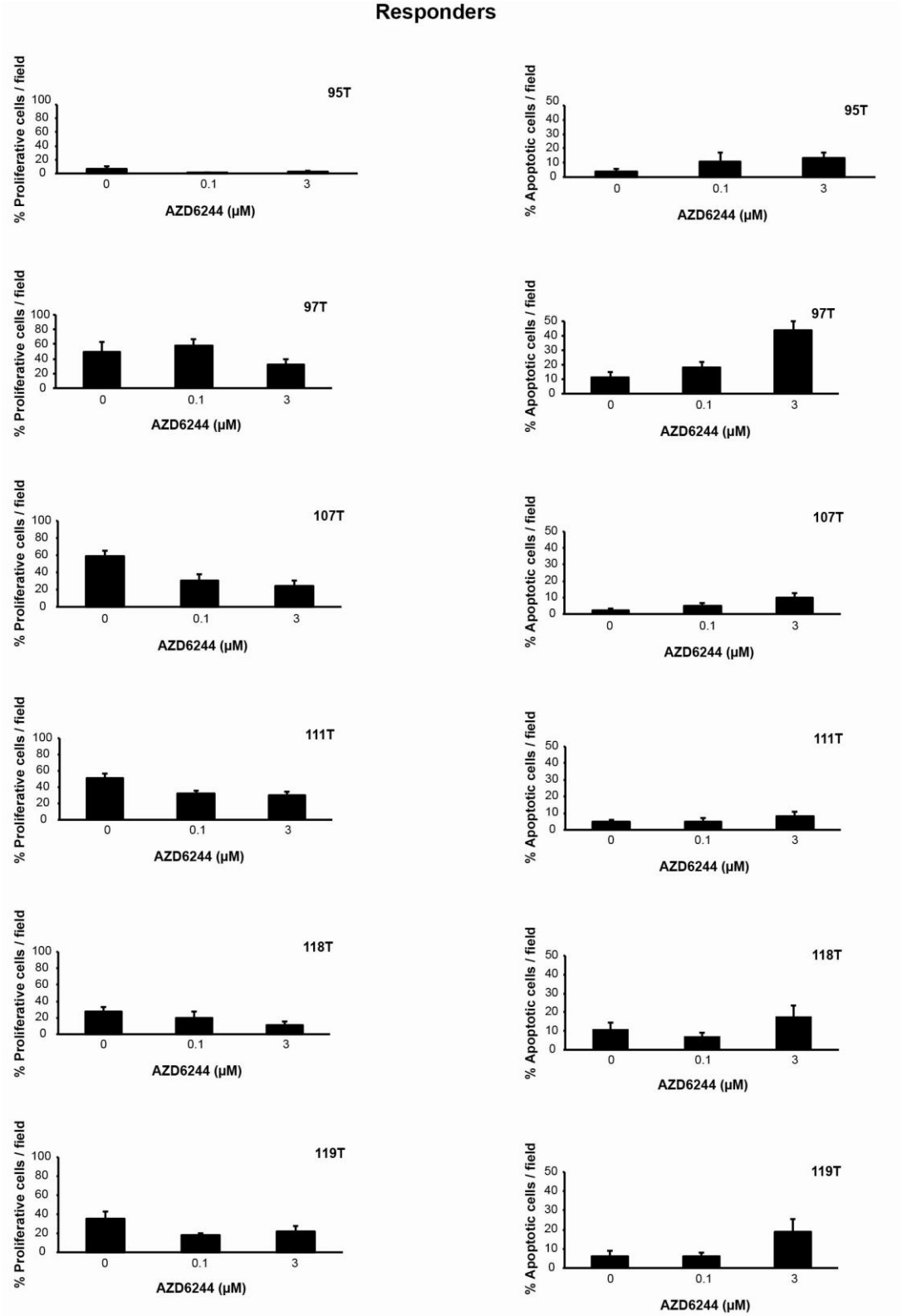


Figure 4.6: Quantitative image analysis of proliferation and apoptosis markers. Tumours were treated with 0-3μM AZD6244 for 1h and immunohistochemistry was performed with antibodies to proliferation marker Ki-67 and apoptosis marker active caspase-3. Five fields of view per sample per treatment were captured at 1000x magnification, and the number of positive cell quantified. Graphs show average of 5 fields of view +/- SE. One tail Student's t test was used to determine p values. Images and graphs show examples of immunohistochemical data obtained from tissue sections from one patient tumour.


Responders
95T
% Proliferative cells / field
100
80
60
40
20
0
0
0.1
3
AZD6244 (μM)
95T
% Apoptotic cells / field
50
40
30
20
10
0
0
0.1
3
AZD6244 (μM)
97T
% Proliferative cells / field
AZD6244 (μM)
97T
% Apoptotic cells / field
AZD6244 (μM)
107T
% Proliferative cells / field
AZD6244 (μM)
107T
% Apoptotic cells / field
AZD6244 (μM)
111T
% Proliferative cells / field
AZD6244 (μM)
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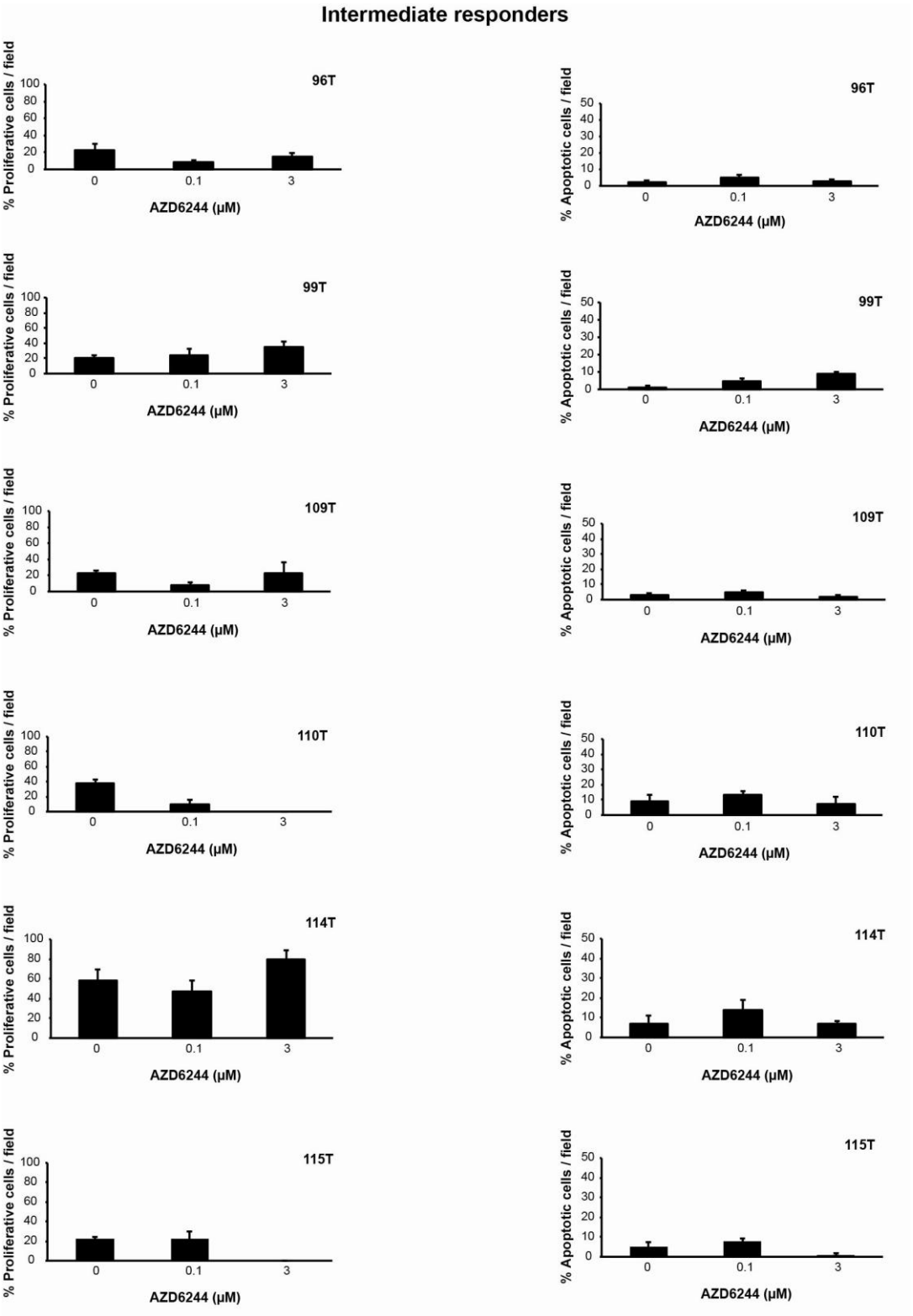
Intermediate responders
96T
% Proliferative cells / field
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80
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3
AZD6244 (μM)
96T
% Apoptotic cells / field
50
40
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20
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0
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0.1
3
AZD6244 (μM)
99T
% Proliferative cells / field
AZD6244 (μM)
99T
% Apoptotic cells / field
AZD6244 (μM)
109T
% Proliferative cells / field
AZD6244 (μM)
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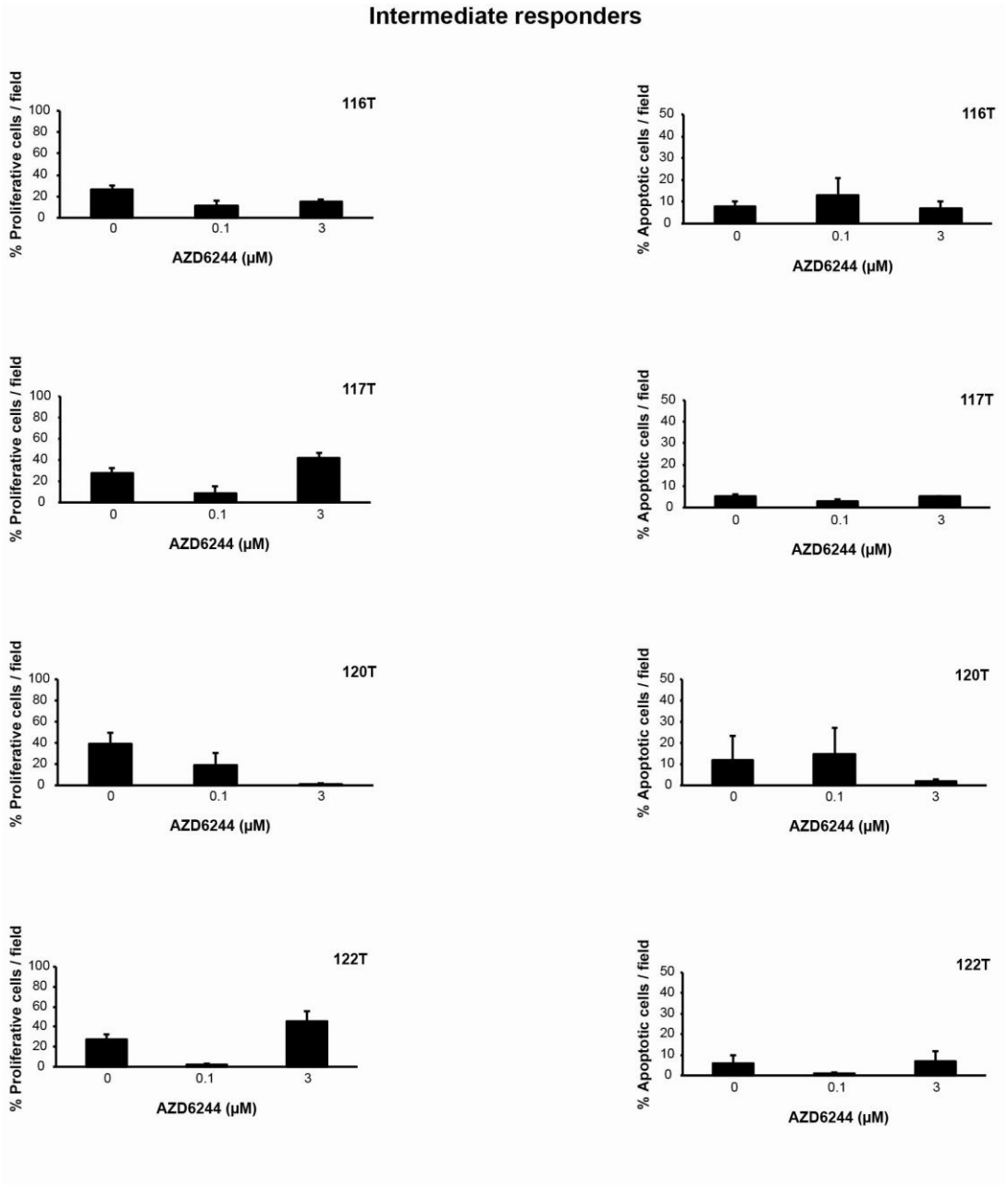
AZD6244 (μM)
115T
% Apoptotic cells / field
AZD6244 (μM)


Intermediate responders
116T
% Proliferative cells / field
100
80
60
40
20
0
0
0.1
3
AZD6244 (μM)
116T
% Apoptotic cells / field
50
40
30
20
10
0
0
0.1
3
AZD6244 (μM)
117T
% Proliferative cells / field
100
80
60
40
20
0
0
0.1
3
AZD6244 (μM)
117T
% Apoptotic cells / field
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40
30
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10
0
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AZD6244 (μM)
120T
% Proliferative cells / field
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AZD6244 (μM)
120T
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AZD6244 (μM)
122T
% Proliferative cells / field
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AZD6244 (μM)
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% Apoptotic cells / field
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0
0.1
3
AZD6244 (μM)

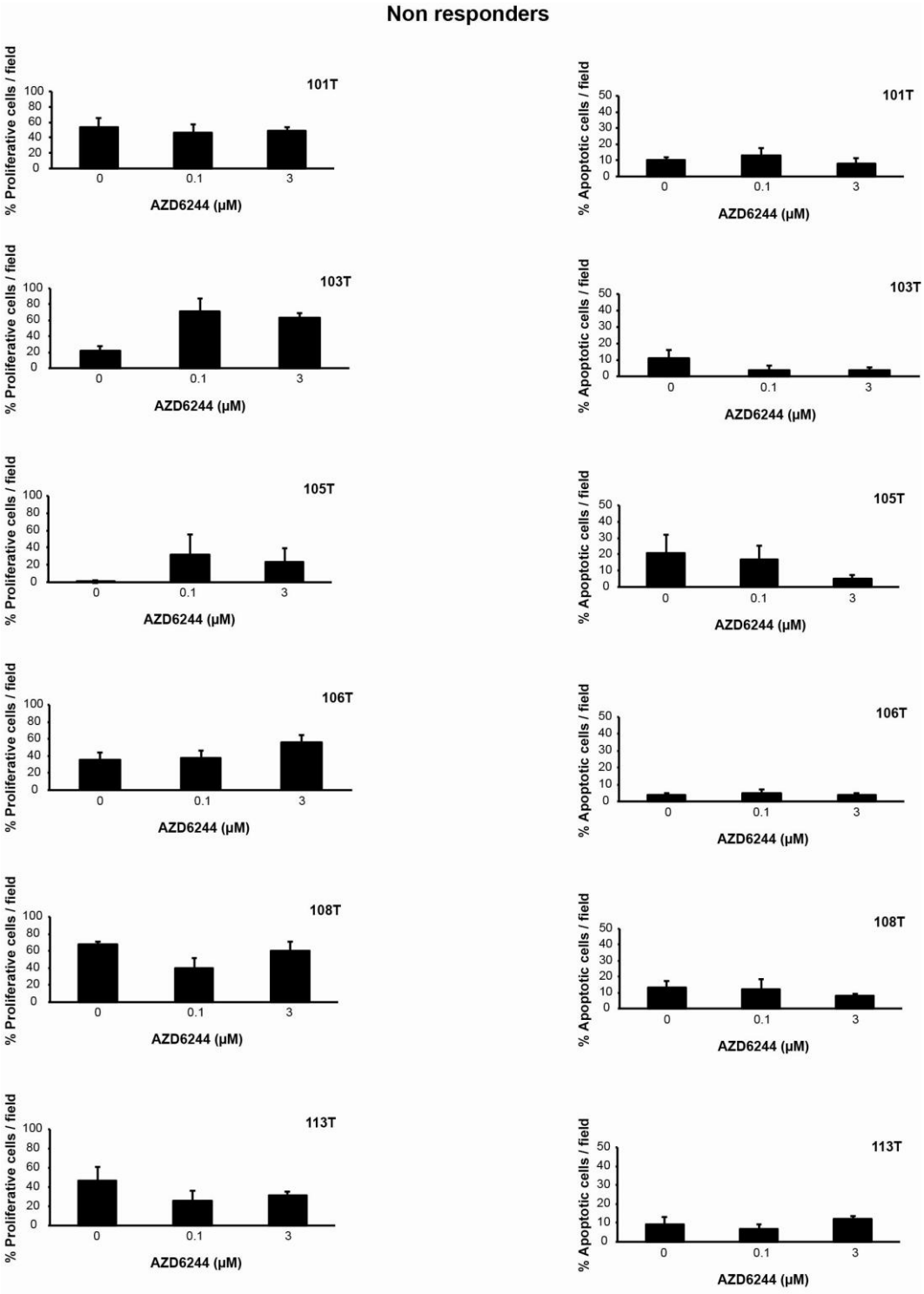


Figure 4.7: AZD6244 effects on proliferation and apoptosis in all tumours treated. Tumours were treated with 0-3μM AZD6244 for 1h and effects on proliferation and apoptosis were measured using immunohistochemical analysis performed as described in Figure 4.6. Data is the average of 5 fields of view +/- SE in each tumour. Different patterns of response to AZD6244 are evident in each of the established response categories.

4.4 - Acute treatment with AZD6244 is sufficient to induce a phenotypic response in human colorectal tumour explants

Analysis of the immunohistochemistry data revealed distinct patterns of response to AZD6244 in colorectal tumours (Table 4.1). Some samples demonstrated a clear dose response for both markers while others showed no response in either marker (Figure 4.7). Using the following criteria, I attempted to stratify the sensitivity of the samples to the agent:

- Tumours that showed a dose-dependent decrease in proliferation and increase in apoptosis, and this change reached 2-fold (+/-20%) for both markers were classified as responders.
- Tumours that presented a response in one marker at both doses or 2 markers only at the lower dose were classified as intermediate responders.
- Tumours that presented no change or an increase in proliferation and decrease in apoptosis were classified as non responders.

According to these criteria, a sample was only classified as a responder if a response was observed in two separate pieces of tissue (wells). This minimised the possibility that the difference in levels of proliferation/apoptosis were a result of inter well variability between tumour pieces. The fact that a response was required in both markers also strengthened the data. For tumours where a response was only observed at the lower dose (intermediate responders) it cannot be ruled out that this response was a consequence of variability in proliferation / apoptosis between tumour pieces.

	Sample ID	IHC Ki-67	IHC Act. Casp3
95T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	0.1	2.8
	3μM AZD6244	0.3	3.3
96T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	0.4	2.5
	3μM AZD6244	0.7	1.5
97T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	1.2	1.6
	3μM AZD6244	0.6	4.0
99T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	1.2	5.0
	3μM AZD6244	1.8	9.0
101T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	0.9	1.3
	3μM AZD6244	0.9	0.8
103T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	3.2	0.4
	3μM AZD6244	2.9	0.4
105T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	32.0	0.8
	3μM AZD6244	24.0	0.2
106T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	1.1	1.3
	3μM AZD6244	1.6	1.0
107T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	0.5	2.5
	3μM AZD6244	0.4	5.0
108T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	0.6	0.9
	3μM AZD6244	0.9	0.6
109T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	0.3	1.7
	3μM AZD6244	1.0	0.7

	Sample ID	IHC Ki-67	IHC Act. Casp3
110T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	0.3	1.4
	3μM AZD6244	0.0	0.8
111T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	0.6	1.0
	3μM AZD6244	0.6	1.6
113T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	0.6	0.8
	3μM AZD6244	0.7	1.3
114T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	0.8	2.0
	3μM AZD6244	1.4	1.0
115T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	1.0	1.6
	3μM AZD6244	0.0	0.2
116T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	0.4	1.6
	3μM AZD6244	0.6	0.9
117T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	0.3	0.6
	3μM AZD6244	1.5	1.0
118T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	0.7	0.6
	3μM AZD6244	0.4	1.6
119T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	0.5	1.0
	3μM AZD6244	0.6	3.2
120T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	0.5	1.3
	3μM AZD6244	0.0	0.2
122T	0μM AZD6244	1.0	1.0
	0.1μM AZD6244	0.1	0.2
	3μM AZD6244	1.7	1.2

Table 4.1: AZD6244 effect on tumour proliferation and apoptosis. Table shows the levels of proliferation marker Ki-67 and apoptosis marker active caspase-3 in patients tumours treated with 0, 0.1 and 3μM AZD6244 for 1h, obtained by immunohistochemical analysis. Colour scheme represents the changes observed in the levels of these markers: Green indicates a >2-fold decrease in proliferation or increase in apoptosis; Yellow indicates a 2-fold (+/- 20%) variation in the levels of Ki-67 and active caspase-3; and Red indicates that no changes or an increase in proliferation and decrease in apoptosis were observed in these tumours.

Using these criteria I found that 6 samples showed an acute phenotypic response to AZD6244, ten tumours presented an intermediate response, and seven tumours did not respond to treatment with this agent (see Table 4.1 and Figure 4.8).

When the data for patients in each of these groups were combined, clear patterns of response to the agent emerged (Figure 4.9). For the responding group there was a dose-dependent decrease in proliferation which reached significance for both doses ($p=0.04$ for 0.1μM and 0.003 for 3μM). There was also a dose-dependent increase in apoptosis which reached significance at the 3μM dose ($p=0.02$). For the intermediate group there was a significant decrease in proliferation ($p=0.0007$) and increase in apoptosis ($p=0.001$) at the 0.1μM dose. P values were calculated using one tail Student's t test. However, minimal response was observed at the 3μM dose. For the non-responders there was no significant change in proliferation / apoptosis.

Due the small nature of the tumours, I could not routinely set up replicates for the treatments. One tumour that I did treat in triplicate showed poor correlation between the replicates (Figure 4.10). However, this tumour was much larger in size (>10x) when compared to the other tumours which could potentially result in morphologic and homeostatic differences between the tumour pieces treated. These data highlight the importance of tumour heterogeneity, suggesting that different areas of the same tumour present differential sensitivity to AZD6244. Given that the combined data of all replicates is not representative of individual response, this tumour samples was not included in the data analysis.

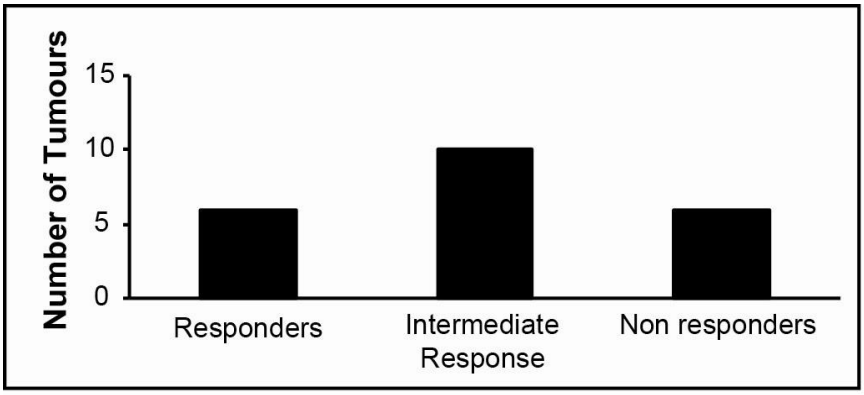


Figure 4.8: Tumour distribution based on sensitivity to AZD6244. Graph shows the number of samples in each response category established according to phenotypic response to treatment with 0.1 and 3μM AZD6244.

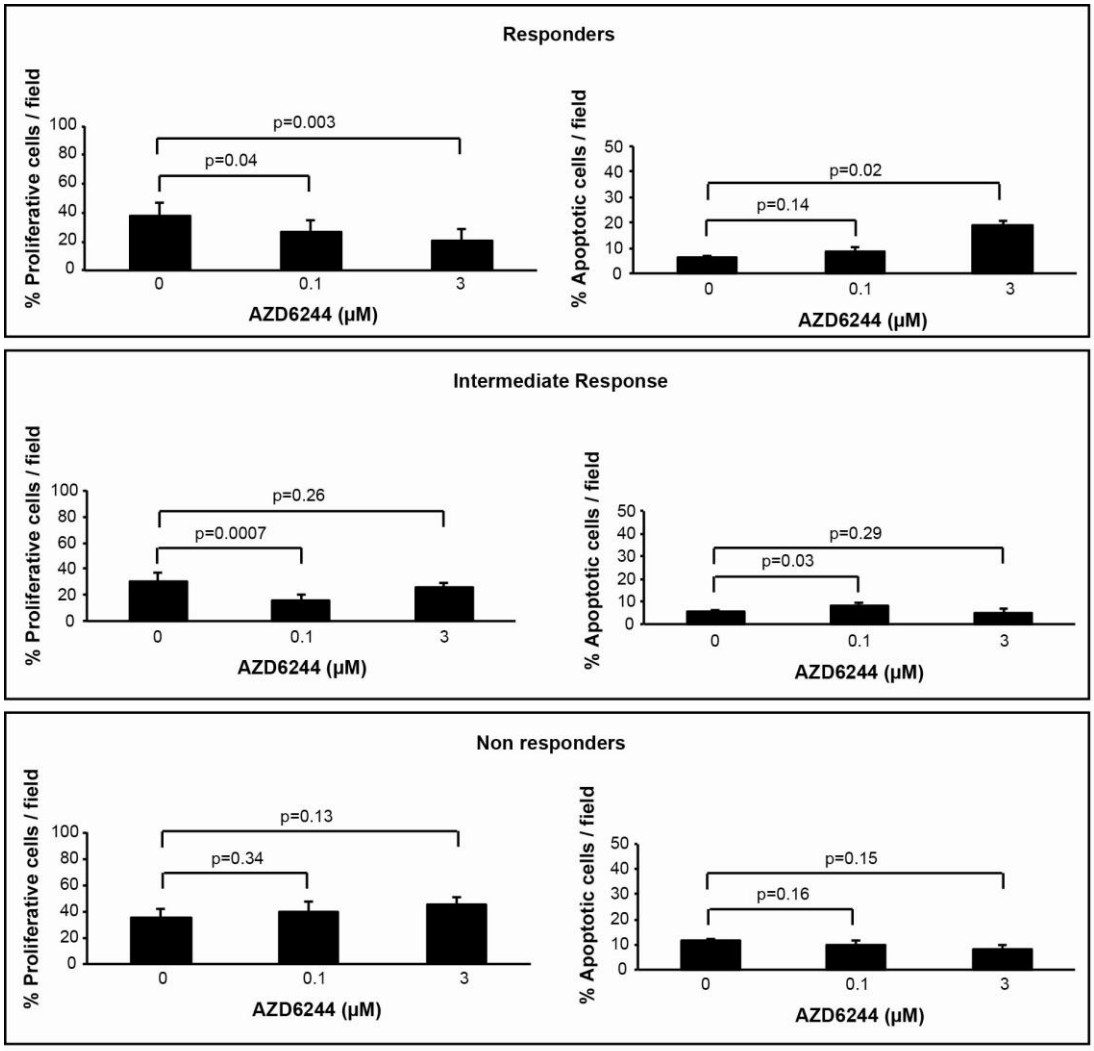


Figure 4.9: Pattern of AZD6244-induced changes in proliferation and apoptosis in each response group. Tumours were treated with 0, 0.1 and 3μM AZD6244, and effects on proliferation and apoptosis were evaluated using immunohistochemical analysis. Quantification of levels of expression of each marker in each individual tumour were performed as described in Figure 4.6. Graphs show the average levels of proliferation marker Ki-67 and apoptosis marker active caspase-3 in each response group. Data were pooled from all tumours of the same response group (Responders, n=6, +/- SE; Intermediate, n=10, +/-SE; Non responders, n=6, +/-SE). One tail Student's t test was used to determine p values.

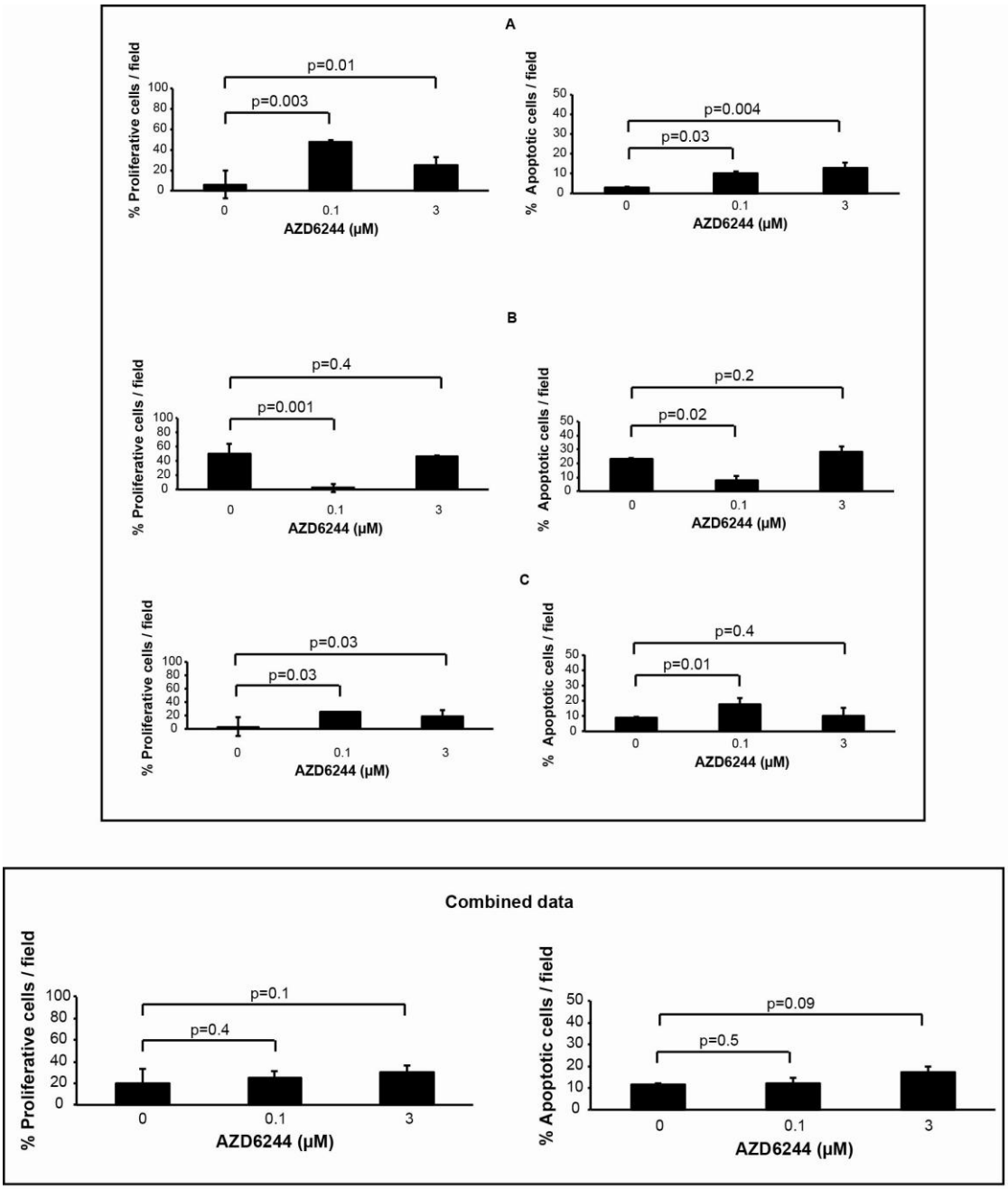


Figure 4.10: AZD6244 effects on proliferation and apoptosis in a patient tumour treated in triplicate. A single tumour was divided into multiple fragments and treated in triplicate. Immunohistochemistry was used to determine AZD6244 effects on proliferation and apoptosis as described in Figure 4.6. Top graphs show data obtained with each individual set of samples. Data shows inconsistent results between the replicates though they all present intermediate response to AZD6244. However, pooled data from the 3 sets of treatments shown in the bottom graphs shows a response pattern characteristic of the non responsive group. One tail Student's t test was used to determine p values.

Analysis of the above data revealed that there was considerable variability in the initial rates of proliferation and apoptosis between tumours (T0 samples). There was also variability in the maintenance of sample integrity when placed in culture (comparing T0 samples to 0μM controls). Therefore, I hypothesised that this variability might account for differences in response to treatment. However, when I compared the expression of Ki-67 and active caspase-3 at T0 in the three groups I found that there was no association between initial rates of proliferation and/or apoptosis and tumour response. Similarly, I found that there was no association between maintenance of tissue viability in culture and response to AZD6244 (Figure 4.11a/b). This excludes any bias introduced by the culturing system.

Taken together, these data suggest that acute treatments with AZD6244 can be used to identify samples with hypersensitivity to this agent.

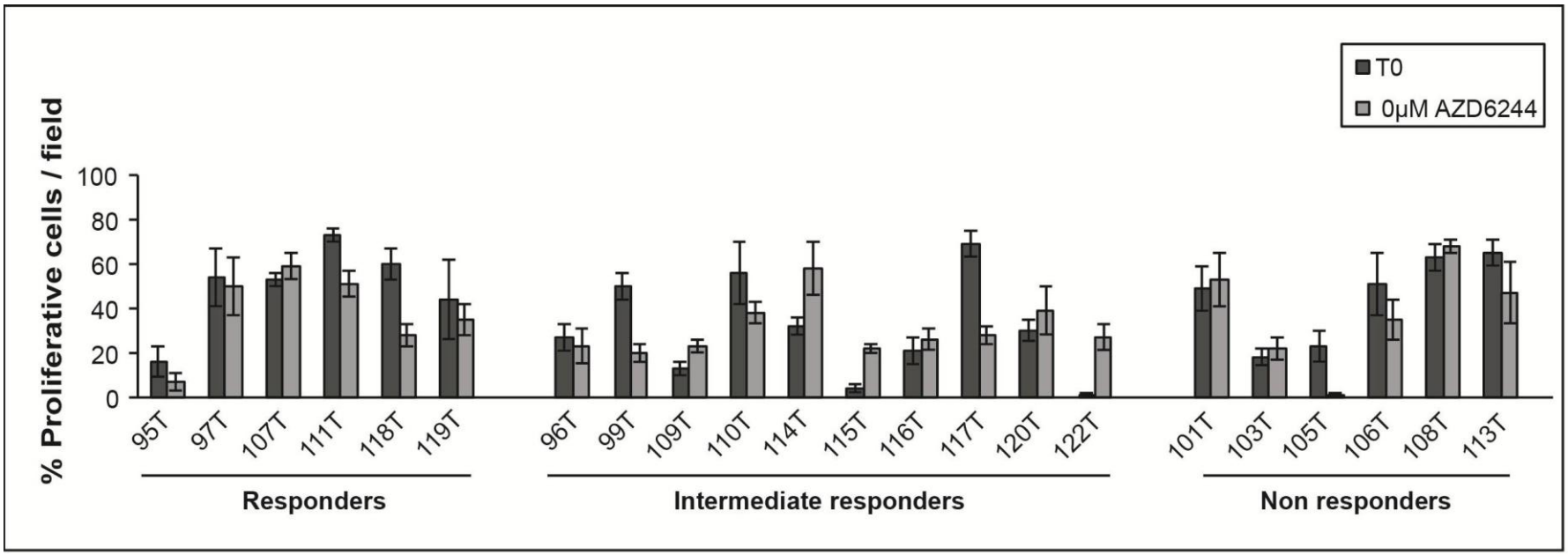


Figure 4.11a: Tissue viability at T0 and while in culture does not correlate with pattern of response to AZD6244. Graph shows the percentage of proliferative cells per field in T0 and non treated controls (0μM) in tumours from each response category, obtained by immunohistochemical analysis as described in Figure 4.6. There are variable initial proliferative rates, and variable effects of culturing conditions on proliferation. However, neither of these were associated with response. One tail Student's t test was used to calculate the p value between responding and non responding tumours, $p>0.05$.

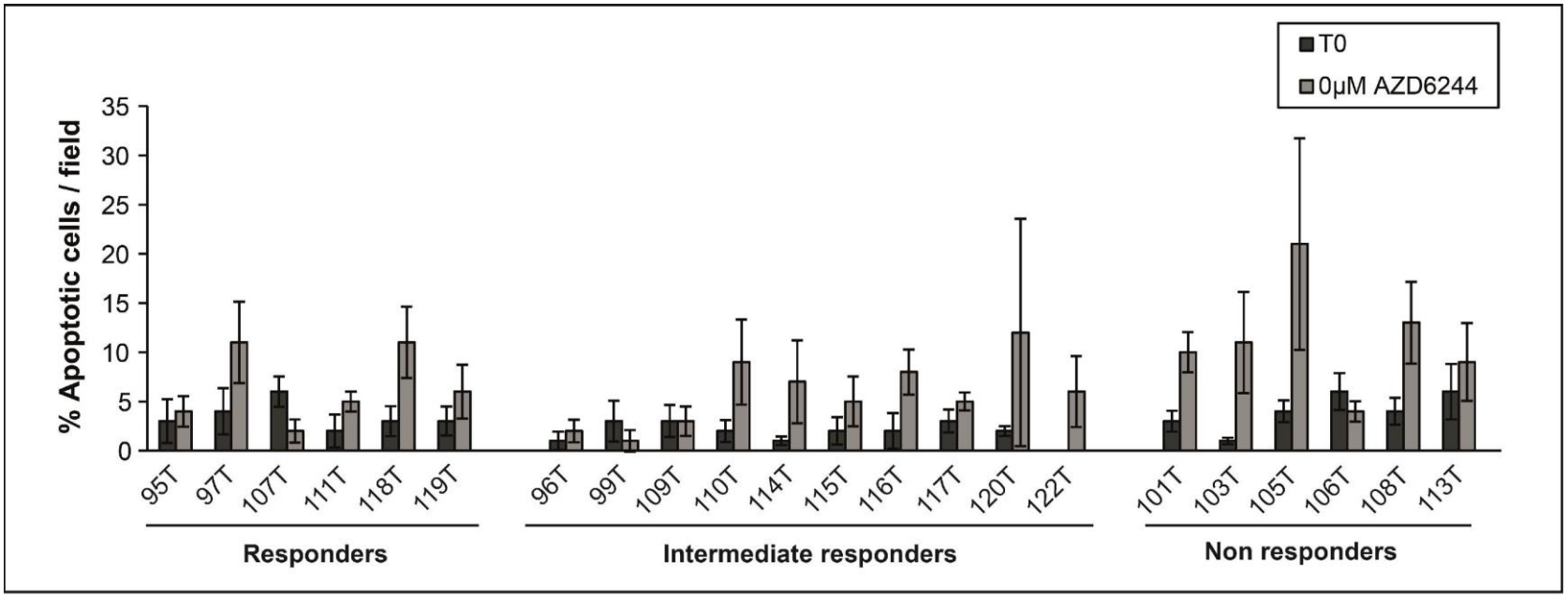
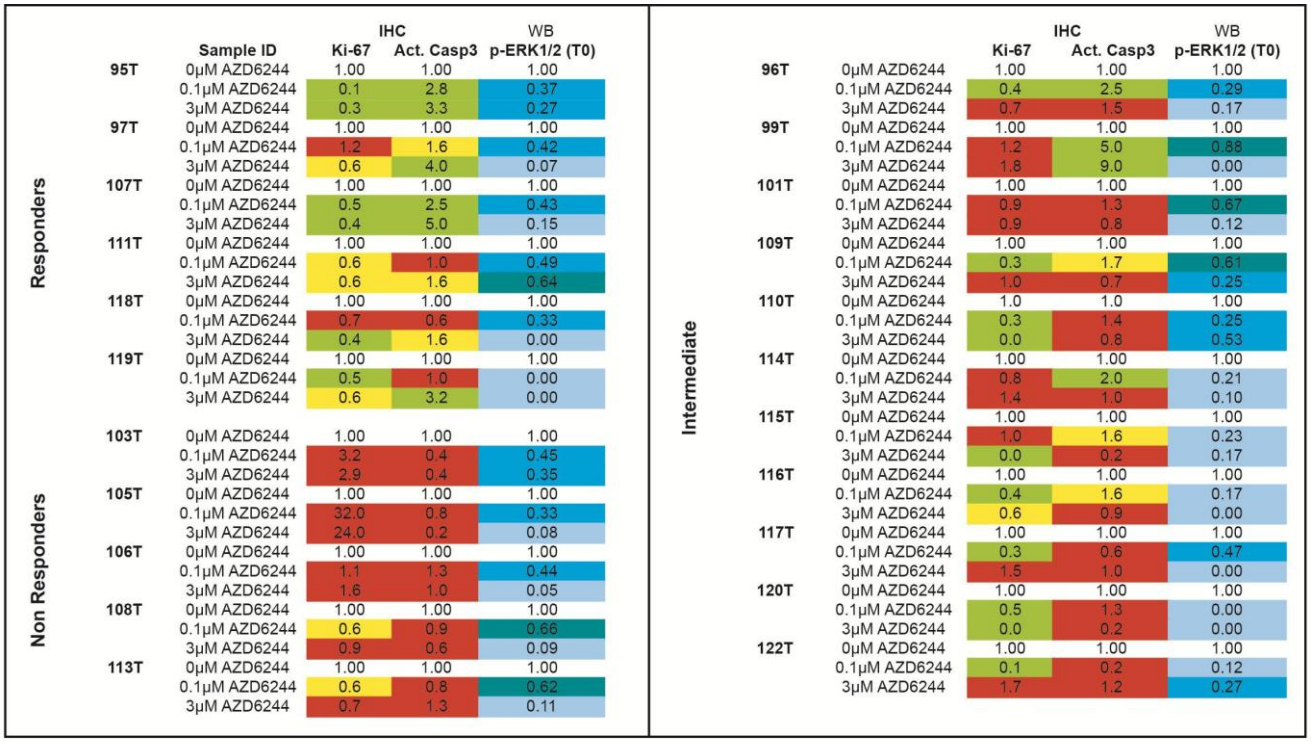


Figure 4.11b: Tissue viability at T0 and while in culture does not correlate with pattern of response to AZD6244. Graph shows the percentage of apoptotic cells per field in T0 and non treated controls (0μM) in tumours from each response category, obtained by immunohistochemical analysis as described in Figure 4.6. There are variable initial apoptosis rates, and variable effects of culturing conditions on apoptosis. However, neither of these were associated with response. One tail Student's t test was used to calculate the p value between responding and non responding tumours, $p>0.05$.

4.5 - Inhibition of ERK1/2 phosphorylation cannot be correlated with phenotypic response

Previous reports indicated that a phenotypic response to AZD6244 correlates with levels of p-ERK1/2 inhibition in xenograft models (*94*) . Therefore, I next compared AZD6244 effects on tumour levels of Ki-67 and active caspase-3 with effects on p-ERK1/2. AZD6244-induced changes in levels of proliferation and apoptosis previously determined (see section 4.4) were compared with p-ERK1/2 inhibition to ascertain if there is a correlation between pharmacodynamic effects and phenotypic response. Correlation coefficients were calculated between normalised expression values of p-ERK1/2 and Ki-67, and p-ERK1/2 and active caspase-3.

As may be expected, given that in the responding group, all markers change in response to AZD6244, a strong positive correlation was observed between effects on p-ERK1/2 and Ki-67 in all six samples, and a negative correlation was observed between effects on p-ERK1/2 and apoptosis in 5 samples. However we also found that p-ERK1/2 decreased to the same extent in samples classified as intermediate and non-responders. This would suggest that the levels of p-ERK inhibition cannot predict a proliferative or apoptotic response to this agent in these primary samples (Table 4.2).



			IHC		WB
		Sample ID	Ki-67	Act. Casp3	p-ERK1/2 (T0)
Responders	95T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	0.1	2.8	0.37
		3μM AZD6244	0.3	3.3	0.27
	97T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	1.2	1.6	0.42
		3μM AZD6244	0.6	4.0	0.07
	107T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	0.5	2.5	0.43
		3μM AZD6244	0.4	5.0	0.15
	111T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	0.6	1.0	0.49
		3μM AZD6244	0.6	1.6	0.64
	118T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	0.7	0.6	0.33
		3μM AZD6244	0.4	1.6	0.00
	119T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	0.5	1.0	0.00
		3μM AZD6244	0.6	3.2	0.00
Non Responders	103T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	3.2	0.4	0.45
		3μM AZD6244	2.9	0.4	0.35
	105T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	32.0	0.8	0.33
		3μM AZD6244	24.0	0.2	0.08
	106T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	1.1	1.3	0.44
		3μM AZD6244	1.6	1.0	0.05
	108T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	0.6	0.9	0.66
		3μM AZD6244	0.9	0.6	0.09
	113T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	0.6	0.8	0.62
		3μM AZD6244	0.7	1.3	0.11

			IHC		WB
			Ki-67	Act. Casp3	p-ERK1/2 (T0)
Intermediate	96T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	0.4	2.5	0.29
		3μM AZD6244	0.7	1.5	0.17
	99T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	1.2	5.0	0.88
		3μM AZD6244	1.8	9.0	0.00
	101T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	0.9	1.3	0.67
		3μM AZD6244	0.9	0.8	0.12
	109T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	0.3	1.7	0.61
		3μM AZD6244	1.0	0.7	0.25
	110T	0μM AZD6244	1.0	1.0	1.00
		0.1μM AZD6244	0.3	1.4	0.25
		3μM AZD6244	0.0	0.8	0.53
	114T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	0.8	2.0	0.21
		3μM AZD6244	1.4	1.0	0.10
	115T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	1.0	1.6	0.23
		3μM AZD6244	0.0	0.2	0.17
	116T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	0.4	1.6	0.17
		3μM AZD6244	0.6	0.9	0.00
	117T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	0.3	0.6	0.47
		3μM AZD6244	1.5	1.0	0.00
	120T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	0.5	1.3	0.00
		3μM AZD6244	0.0	0.2	0.00
	122T	0μM AZD6244	1.00	1.00	1.00
		0.1μM AZD6244	0.1	0.2	0.12
		3μM AZD6244	1.7	1.2	0.27

Table 4.2: Pharmacodynamic effects of AZD6244 do not always translate into phenotypic response. Table shows the effects of treatment with AZD6244 on the expression of Ki-67 and active caspase-3, and on p-ERK1/2 expression. Dose-dependent inhibition of p-ERK1/2 occured in 21 out of 22 samples treated with AZD6244, but only a small group of samples suffered a dose-dependent inhibition of proliferation and induction of apoptosis. Colour scheme represents the changes observed in the expression of these markers: Green indicates a >2-fold decrease in proliferation or increase in apoptosis; Yellow indicates a 2-fold (+/-20%) variation in the expression of Ki-67 and active caspase-3; and Red indicates that no changes or an increase in proliferation and decrease of apoptosis were observed in these tumours. Blue indicates the extent of p-ERK1/2 inhibition, with lighter shades representing the highest levels of inhibition.

4.6 – Further characterisation of patient tumour response to AZD6244

To further characterise the patient tumour response to treatment with AZD6244 I also investigated the levels of other molecules known to be involved in the response to this agent.

In addition to p-ERK1/2 inhibition, increased p21 and decreased cyclinD1 levels have been associated with response to AZD6244 (*95, 114*). I analysed the expression of these markers in the first thirteen tumour explants treated. From this panel of samples, five tumours showed increased p21 expression in response to the agent (Figure 4.12a). However, this did not appear to be related with response as it was apparent in tumours classified as responders and non responders to treatment. I therefore proceeded to investigate expression of p27, a cell cycle control protein known to inhibit Cyclin D1/CDK4 and cause G1 phase cell cycle arrest (*220*) that has also been associated with response to AZD6244 treatment (*95*). I used western blot analysis to investigate levels of p27 in 9 of the above colorectal tumour samples. This panel included 2 tumours classified as responders, 5 tumours showing intermediate response, and 2 non responders. I found that p27 expression was only increased in 3 tumours treated with 0.1μM AZD6244 (116T, 117T and 118T). Therefore, I was unable to identify a pattern of expression of p27 related to tumour response to AZD6244 (Figure 4.12b).

Cyclin D1 expression was also evaluated in the initial panel of thirteen tumours but the expected decrease was not observed in any sample (data not shown). In some samples, Cyclin D1 signal was very weak, even at T0, making it difficult to draw conclusions regarding effects of AZD6244 on levels of the protein.

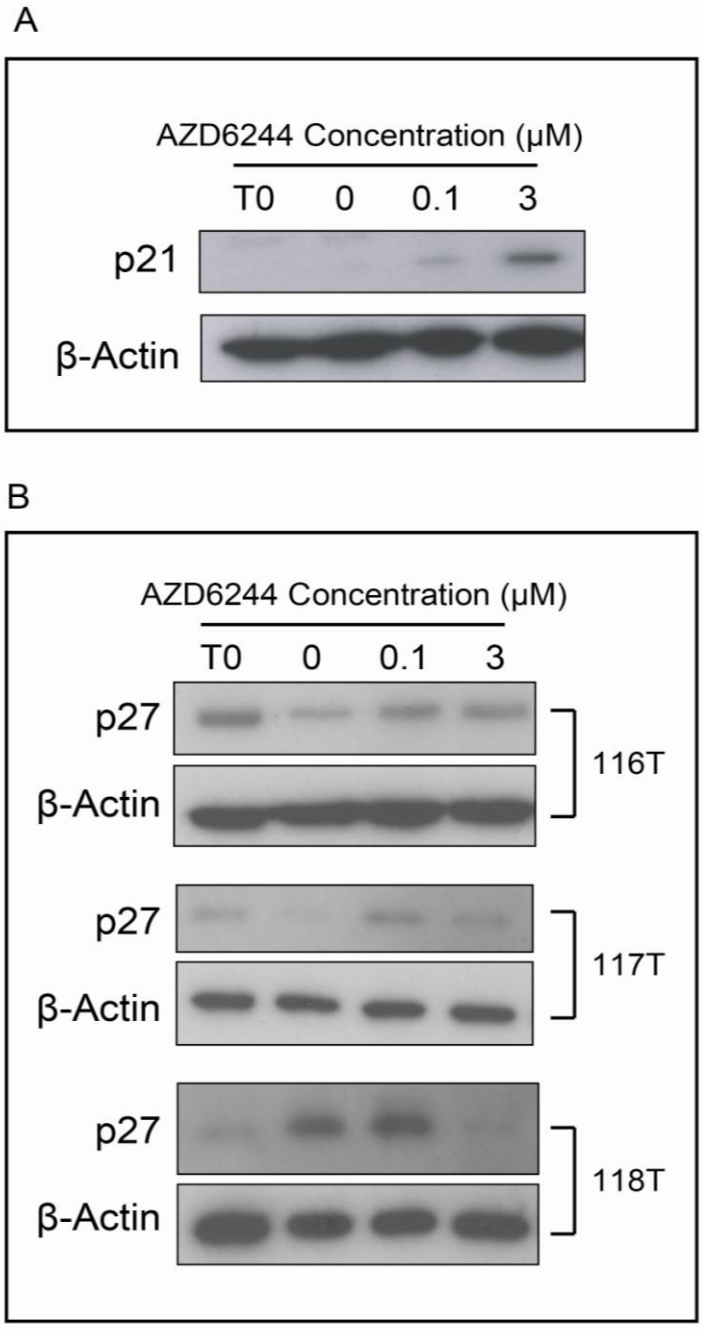


Figure 4.12: AZD6244 causes cell cycle arrest in patient tumours. Figure shows western blot analysis of p21 levels (A) in one tumour treated with AZD6244 for 1h, and p27 levels (B) in three tumours treated with the same regime. Increased p21 and p27 levels in treated samples suggests induction of cell cycle arrest in response to AZD6244.

I then looked at the expression of CDK4 in the remaining ten tumour samples but again AZD6244 effects on this marker could not be measured due to weakness of western blot signal observed in the majority of the samples.

These data suggests that patterns of response to AZD6244 shown in cell lines might not translate to patient tumours where a more complex network of events seems to be involved in the response to treatment with this agent.

4.7 – Response to aspirin can be measured with acute treatments

Having shown that acute treatment of human tumour explants can be used to study the response to AZD6244, I hypothesised that this model is also useful to study the response to other therapeutic agents.

Work in the host lab has been aimed at understanding the mechanisms by which aspirin and related agents act against colon cancer cells. However, these studies have mainly been performed in colorectal cancer cell lines and xenografts, and the significance to human tumours has not yet been established. In these models, aspirin was shown to induce activation of the NFκB pathway, repression of NF-κB-driven transcription and consequently, the induction of apoptosis (*134, 221*). In this setting, aspirin was also reported to induce Cyclin D1 degradation and cell cycle arrest upstream of effects on NF-κB (*135*). In more recent studies, the lab has demonstrated that an early response to aspirin is modulation of nucleolar structure and function. In particular, they have demonstrated that aspirin causes a decrease in levels of the critical component of the PolI transcription machinery, TIF-1A. Furthermore, this decrease happens as a very early response to the agent.

Given the novelty of this new data, I set out to determine whether the effects observed in colorectal cancer cells could be detected using acute treatment of human colorectal tumour explants. I treated 8 tumour samples with 0 and 100μM aspirin (which is the expected serum salicylate level after taking one 300mg tablet) and performed western blot analysis, to investigate levels of Cyclin D1, CDK4 and TIF-1A. Immunohistochemical analysis was also performed on fixed tissue to investigate the effects of this agent on proliferation and apoptosis.

I found that levels of both Cyclin D1 and CDK4 were very low, even in non treated controls (0μM), and that aspirin had minimal effect on the expression of either marker (data not shown). In contrast, I found that in 5/7 patient tumours analysed, aspirin caused a significant decrease in levels of TIF-1A, in keeping with data obtained from colorectal cancer cells (Figure 4.13).

Quantitative analysis of tissue sections stained for Ki-67 and active caspase-3 was carried out on 5 fields of view as described in section 4.3. I found that aspirin effects on proliferation and apoptosis were minimal, and variable between tumours (Figure 4.14).

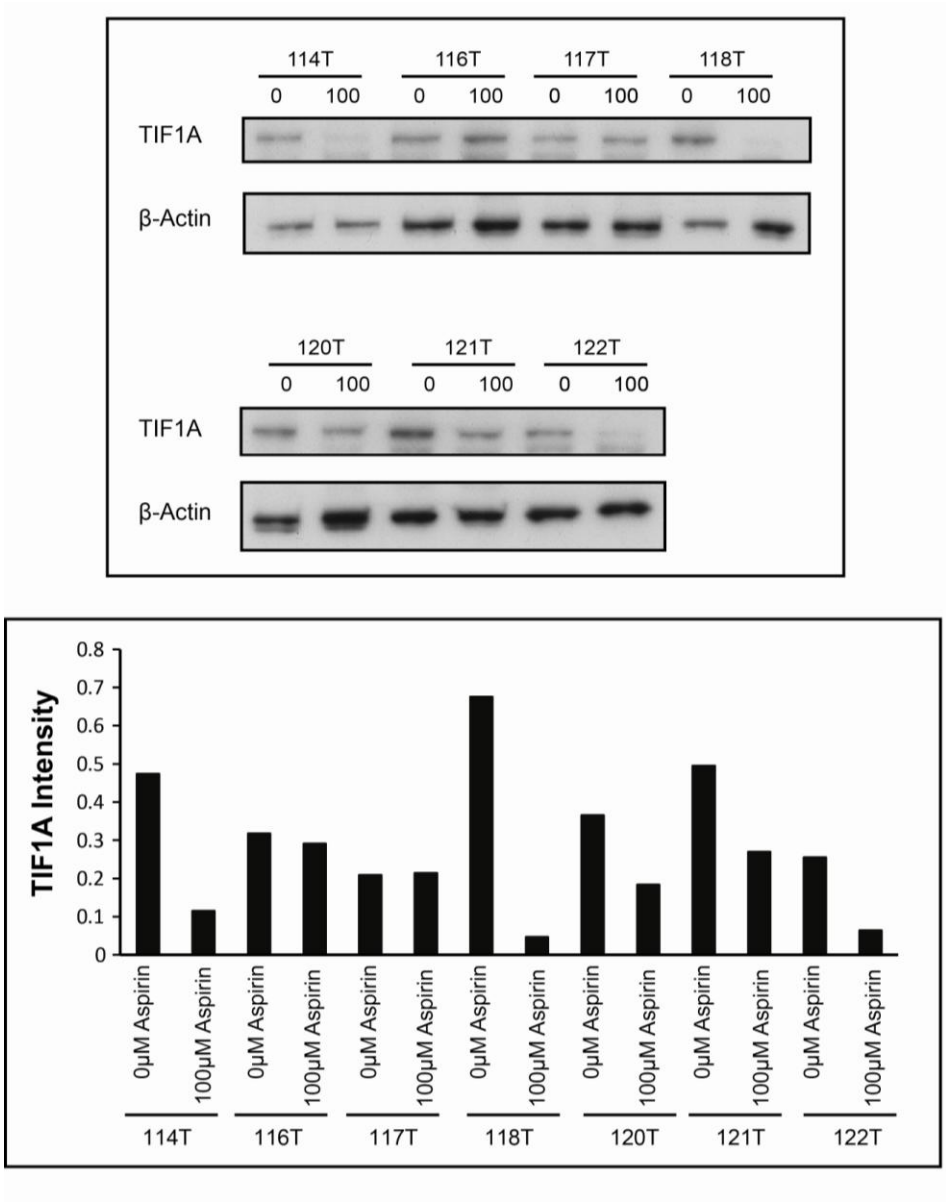


Figure 4.13: Aspirin reduces TIF1A levels in patient tumours. Figure shows western blot analysis of the levels of TIF1A in tumours treated with 0 and 100μM aspirin for 1h. ImageJ was used to quantify blot intensities. TIF1A levels were normalised with β-Actin used as loading control. Data shows that TIF1A levels were reduced in 5/7 tumours as a result of aspirin treatment. Western Blotting was performed by Kathrin Kennerknecht.

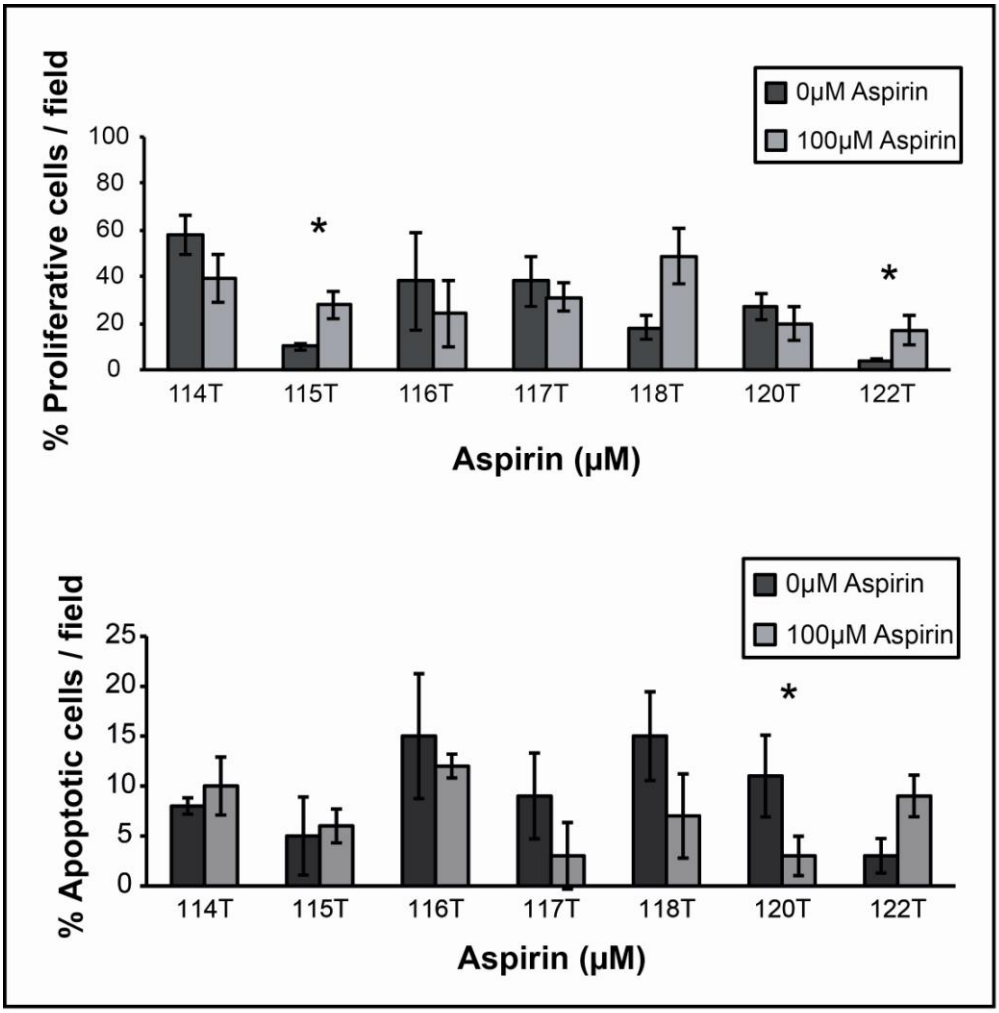


Figure 4.14: Aspirin effects on tumour proliferation and apoptosis. Seven tumours were treated with 0 and 100μM aspirin for 1h. Effects on proliferation and apoptosis were measured by immunohistochemical analysis with antibodies for Ki-67 and active caspase-3. Five fields of view per sample per marker were captured, then the number of positive cells counted and the average between the 5 fields calculated. Data are the average of 7 tumours +/- SE. One tail Student's t test was used to calculate p values. Asterisk (*) indicates p≤5. Graphs show that aspirin effects on proliferation and apoptosis are not consistent between tumours, and a pattern of phenotypic response to this agent could not detected.

Discussion:

The data here presented demonstrate that by using a short term treatment regime, tumours showing hypersensitivity to the MEK1/2 inhibitor AZD6244 can be identified. The percentage of tumours found to be sensitive (27%) was similar to that observed by Adjei *et al* (*115*) who, in clinical studies, also observed a more than 2-fold inhibition of proliferation in 25% of solid tumours from patients recruited for clinical trial. This suggests that this model can provide an indication of tumour sensitivity to AZD6244 and that it could therefore be used to identify patients more likely to respond to treatment with this agent.

Balmanno *et al* (*114*) have previously demonstrated that sensitivity to AZD6244 does not correlate with p-ERK1/2 inhibition, in colon cancer cell lines. In fact, I found that p-ERK1/2 was inhibited in all tumours within 1h of treatment with AZD6244 regardless of phenotypic response. These data are also in keeping with that of Adjei *et al* (*115*) who found that despite full inhibition of ERK1/2 phosphorylation within one hour of treatment, effects on tumour proliferation were only observed in a subset of patients; and that of Davies *et al* (*94*) who reported similar observations with mouse xenografts.

In order to analyse the data, I established a system to categorise the tumours into responders and non responders. To be categorised as a responder, tumours were required to show a more than 2-fold (+/- 10%) increase in apoptosis and decrease in proliferation at both 0.1 and 3μM AZD6244. As this involved response in two separate pieces of the tumour, this categorisation contributed to remove the potential that the differences in levels of proliferation / apoptosis were due to tumour

heterogeneity. However, it excluded tumours that only responded at a lower dose, which may be a very important group (see Chapter 5).

From the group of non responder tumours, those that showed a lack of AZD6244-induced effect on proliferation and apoptosis, some presented increased p21 levels after 1h treatment with the agent. One possibility is that some non responder tumours quickly underwent cell cycle arrest, but have not been exposed to the agent long enough for apoptotic response to be induced. This was previously reported by Balmanno *et al* (*114*) in HCT116 cells in which cell cycle arrest was observed shortly after exposure to AZD6244, but effects on apoptosis could only be measured after 18h of treatment. However, conclusions could not be drawn based on p21 levels only as, in some cells lack of expression of this marker is associated with homozygous p53 mutation (*222*). Therefore, the lack of p21 increase in some of the tumours analysed cannot be related to drug response without determining p53 status.

Using this treatment regime, I was restricted to examining tumour response to AZD6244 within one hour of treatment. However, Davies *et al* (*94*) reported AZD6244-induced apoptosis 8 h after treatment in mouse xenografts; and Balmanno *et al* (*114*) reported this effect after 18h of treatment in more sensitive cells, and lack of effect on apoptosis in resistant cell lines even after 72h of exposure to the drug. This would suggest that the tumours I identified as responders presented hypersensitivity to AZD6244.

My findings showing that HCT116 cells have increased sensitivity to AZD6244 are in keeping with those of Davies *et al* and Balmanno *et al* (*94*, *114*). Importantly, I found that AZD6244 induced apoptosis in HCT116 cells within 1h of treatment. Though such an early apoptotic response has not been observed by other

authors, it provided good indication that a phenotypic response to AZD6244 could be detected using acute (short time point) treatment of patient tumours. Data obtained with HRT18 and RKO cells are also in keeping with that of Balmanno *et al* (*114*) regarding intrinsic resistance to AZD6244 and lack of caspase mediated apoptosis. However, a wide range of sensitivities to AZD6244 has been previously observed in colon cancer cell lines.

Data obtained with aspirin also indicates that the acute treatment model might be useful for testing therapeutic response to any agents that rapidly induce a pharmacodynamic response. It was very interesting to find that patient tumours show TIF1A depletion in response to aspirin. I also found that this is not associated with changes in proliferation or apoptosis. However, this is not surprising as in tissue culture systems, changes to TIF1A are observed within 1h of treatment whereas the indication of apoptosis, at the same aspirin dose, is not observed until 16h after treatment. Given that TIF1A is critical for ribosomal biogenesis (*223*), and depletion of this protein has previously been shown to mediate apoptosis (*224*), aspirin effects on TIF1A will undoubtedly contribute to the pro-apoptotic / anti-tumourigenic effects of this agent. Further studies are currently underway in the lab to identify markers for TIF1A response.

In conclusion, I have identified a method of categorising colorectal cancer response to AZD6244 and potentially aspirin. I have also identified tumours that can be used to investigate markers for hypersensitivity to this agent.

Chapter 5: Molecular markers of sensitivity / resistance to AZD6244

Introduction:

AZD6244 has been extensively studied in cell lines and tumour xenografts models, and in clinical trials, but even though the response to this therapeutic agent has been significantly characterised, markers of sensitivity and resistance have not yet been fully established. Markers of resistance to AZD6244 such as the presence of *KRAS* or *BRAF* mutations, activation of the Akt pathway or increased levels of basal Cyclin D1 have been proposed (*94, 114*). However, the significance of these markers to the clinic is still unknown.

My data obtained with patient tumours treated with AZD6244 revealed distinct patterns of response to this agent. Therefore, I set out to investigate if each of the response groups presented distinct molecular signatures that would allow for identification of markers of sensitivity/resistance to AZD6244 in a more clinically relevant context.

The presence of *KRAS* or *BRAF* mutations is a common feature of cancers of different origins. The presence of these mutations leads to a constitutively active MAPK pathway which results in increased expression of genes responsible for tumour proliferation and survival. In colorectal cancer, these mutations occur in 45% and 12 % of tumours, respectively, and are mutually exclusive (*98, 100*). *KRAS* mutations occur predominantly in codons 12 and 13, and less frequently in codons 61, 63 and 146. In colorectal cancers the frequency of these mutations is ~80% and 18% for codons 12 and 13 respectively; and less than 5% for codons 61, 63 and 146 (*225, 226*). The most frequent *BRAF* mutation occurs in exon 15: T1796A by substitution of valine by glutamic acid at position 599, and it's commonly known as V600E mutation (*227, 228*). Given that these mutations lead to hyperactivity of the

ERK1/2 pathway, it has been proposed that tumours harbouring either mutation would present increased sensitivity to MEK1/2 inhibition by AZD6244. I therefore proceeded to evaluate the mutational status of *KRAS* and *BRAF* in my panel of tumours and compare it with pharmacodynamic and phenotypic response to treatment with this agent. Tumours were also tested for *PIK3CA* mutations as the activation of this pathway is known to increase resistance to MEK1/2 inhibition (*229, 230*). *PIK3CA* encodes the p110 catalytic subunit of PI3K, and mutations in this gene lead to elevated Akt activity (*231, 232*). A recently published study performed on the largest European cohort of metastatic colorectal cancers identified *PIK3CA* mutations in 14.5% of patients (*233*).

I also investigated the correlation between phenotypic response to treatment with AZD6244 and levels of markers thought to be involved in the response to this agent such as Cyclin D1 and Akt. Using Reverse Phase Protein Arrays (RPPA). I also attempted to identify molecular signatures of sensitivity and resistance to this agent.

Results:

5.1 - Analysis of *KRAS*, *BRAF* and *PIK3CA* mutational status

To determine mutational status of KRAS and BRAF in my panel of tumours, I initially used Applied Biosystem's BigDye Terminator v.3.1 Cycle Sequencing protocol to sequence PCR products previously generated using primers for all *KRAS* mutations and $BRAF^{V600E}$ mutation. However, after several attempts I concluded that this method was not sensitive enough to detect mutations in samples with high cellular heterogeneity. Mutational analysis was then performed by AstraZeneca.

Using their Roche Cobas system *KRAS* mutations located in codons 12 or 13, $BRAF^{V600E}$ mutation, and *PIK3CA* mutation, were investigated.

5.1.1 - AZD6244 induces p-ERK1/2 inhibition in mutant and wild type tumours

From my panel of samples, 27% of tumours were found to be KRAS mutant, 8% BRAF mutant and 8% carried *PIK3CA* mutations, including one tumour where a *BRAF* mutation was also found.

First, I compared the levels of p-ERK1/2 observed at T0 between wild type, and *KRAS* or *BRAF* mutant tumours. I found that, contrary to what was expected, mutant tumours did not show higher levels of p-ERK1/2 activity at T0 compared to their wild type counterparts (Figure 5.1). There was also no difference in response to AZD6244 between wild type and mutant tumours, which is consistent with the mutational status not affecting basal p-ERK1/2 levels (Figure 5.2). Similar results were obtained with *PIK3CA* mutant tumours in that there was no correlation observed between mutational status, basal levels of p-ERK1/2 or the effects on AZD6244 on the activity of this kinase (Figure 5.2).

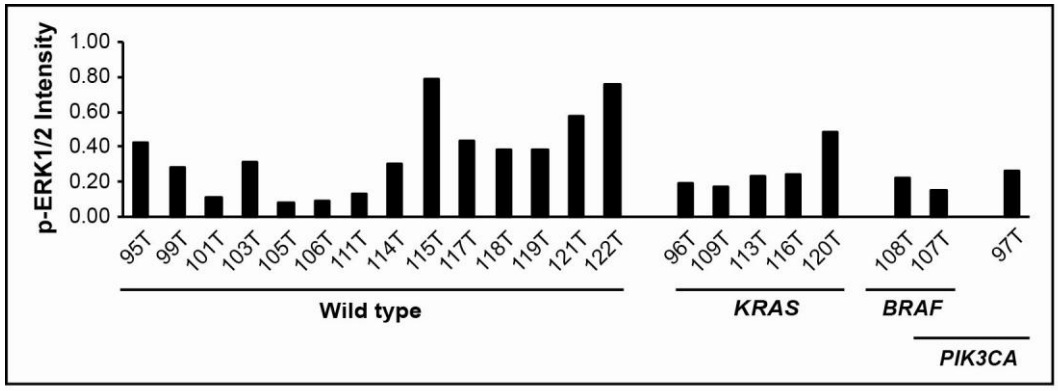


Figure 5.1: Patient tumours carrying *KRAS* or *BRAF* mutations do not present increased ERK1/2 activity. Western blot analysis was performed on whole cell lysates extracted from T0 samples with antibodies to native and phosphorylated ERK1/2. β-actin was used as a loading control. ImageJ was used to quantify blot intensities. Native and phosphorylated ERK1/2 levels were normalised with β-actin, and p-ERK1/2 was normalised with native ERK1/2 protein. Graph shows the expression of p-ERK1/2 at T0 in wild type and *KRAS*, *BRAF* and *PIK3CA* mutant tumours. Contrary to what was expected, tumours carrying *KRAS* or *BRAF* mutations did not show increased p-ERK1/2 activity when compared to wild type tumours.

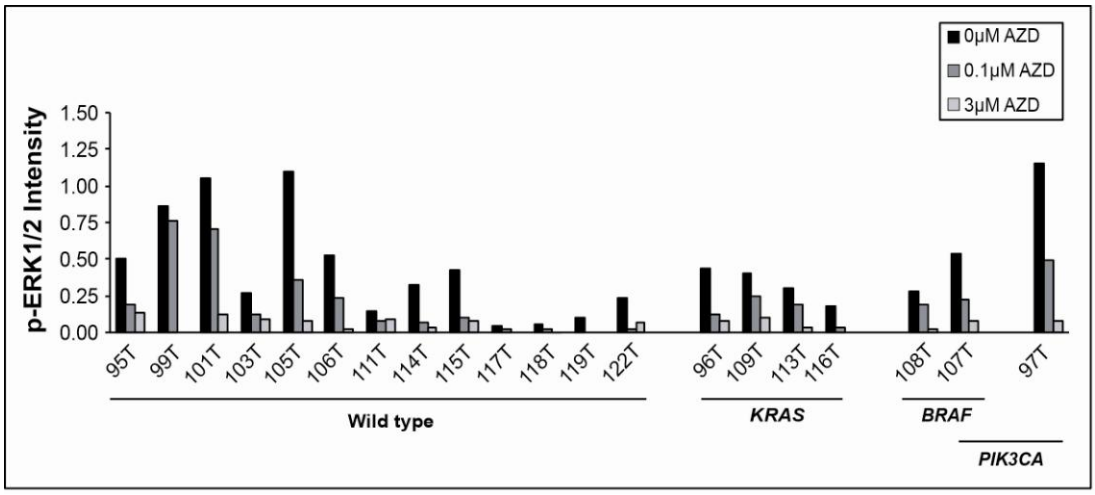


Figure 5.2: *KRAS*, *BRAF* and *PIK3CA* mutational status does not influence AZD6244-induced p-ERK1/2 inhibition. Tumours were treated with 0, 0.1 and 3μM AZD6244 for 1h and western blot analysis was performed as described in Figure 5.1 to investigate effects of this agent on p-ERK1/2 activity. Data shows that p-ERK1/2 is equally inhibited in mutant and wild type tumours.

5.1.2 - *KRAS* and *BRAF* mutational status is not sufficient to predict phenotypic response to treatment with AZD6244

Next, I compared the mutational status of 21 tumours with proliferative / apoptotic response to AZD6244. I found that only two tumours with *KRAS* or *BRAF* mutations showed a dose-dependent inhibition of proliferation (as determined by immunohistochemistry with antibodies to Ki-67 (see section 4.3) (Figure 5.3). Induction of apoptosis was also observed in tumours with mutations in these genes, but was not dose-dependent (Figure 5.4). Therefore, *KRAS* and *BRAF* mutant tumours cannot be classified as responders according to the previously established response criteria (See section 4.3 and Figure 5.5). These data would suggest that the mutational status of these proteins alone cannot be used as an indicator of phenotypic response to AZD6244 in this explant model.

As mutational status may confer increased sensitivity to the drug, I next focussed on the phenotypic response to the lower dose of the agent (0.1uM). Interestingly, I found that all tumours (7/7) carrying these mutations presented inhibition of proliferation in response to low dose treatment with AZD6244, and that in 4 out of 7 tumours this effect was more than 2-fold (Figure 5.6). This is in contrast to wild type tumours where only 7/13 samples showed inhibition of proliferation in response to 0.1μM AZD6244, and from these 7 only 3 displayed a more than 2-fold effect (Figure 5.6). When I looked at the effects of 0.1μM AZD6244 on apoptosis in wild type and mutant tumours I found that response to low dose AZD6244 seemed to be more variable across wild type tumours. However, no further conclusions could be drawn from this data (Figure 5.7).

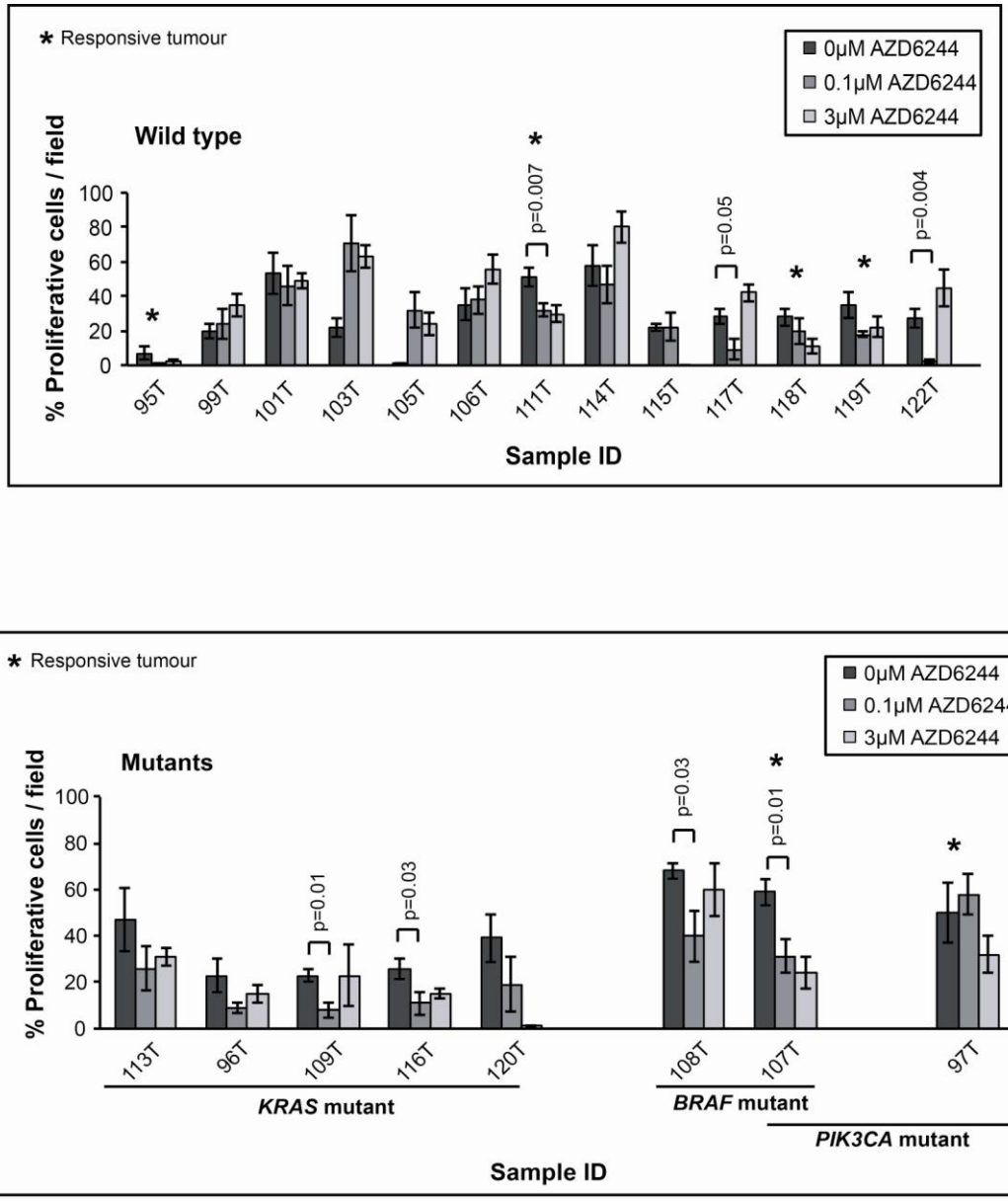


Figure 5.3: AZD6244 effects on proliferation in wild type and *KRAS*, *BRAF* and *PIK3CA* mutant tumours. Tumours were treated with 0, 0.1 and 3μM AZD6244 and effects on proliferation were measured by immunohistochemical analysis with antibodies to proliferation marker Ki-67. Five fields of view were captured per sample and the number of positive cells was counted. Data are the average of 5 fields of view +/- SE. One tail Student's t test was used to calculate p values. Only values of p≤5 are shown. Data suggests that KRAS and BRAF mutant tumours are more sensitive to treatment with 0.1μM AZD6244 than wild type tumours. 4 in 7 mutant tumours showed significant inhibition of proliferation at this concentration, while this was only observed in 2 in 13 wild type tumours. However, this effect was not dose-dependent so these tumours were not classified as responders.

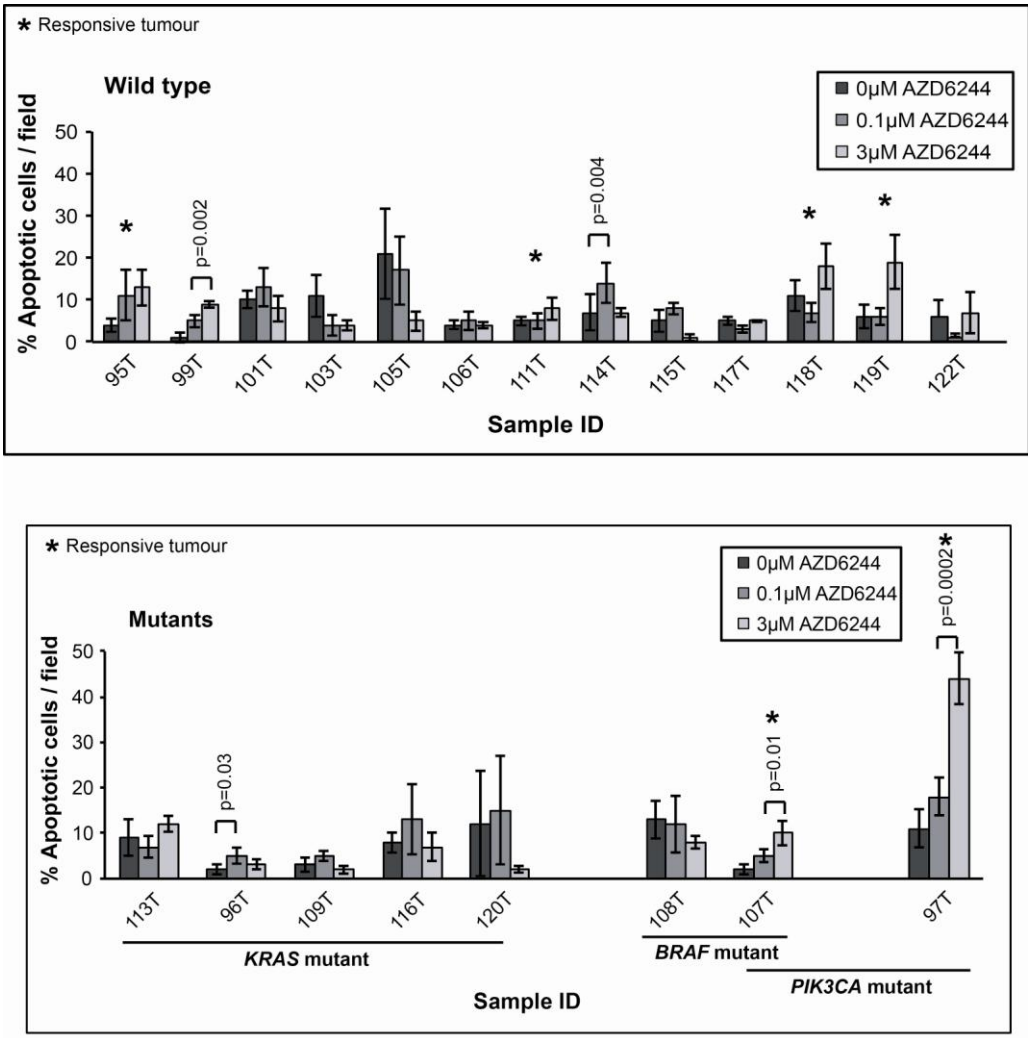


Figure 5.4: AZD6244 effects on apoptosis in wild type and *KRAS*, *BRAF* and *PIK3CA* mutant tumours. Tumours were treated with 0, 0.1 and 3μM AZD6244 and effects on apoptosis were measured by immunohistochemical analysis with antibodies to apoptosis marker active caspase-3. Marker quantification was performed as described in Figure 5.3. Data are the average of 5 fields of view +/- SE. One tail Student's t test was used to calculate p values. Only values of p≤5 are shown. No correlation was found between the presence of either mutation and AZD6244-induced increase in apoptosis.

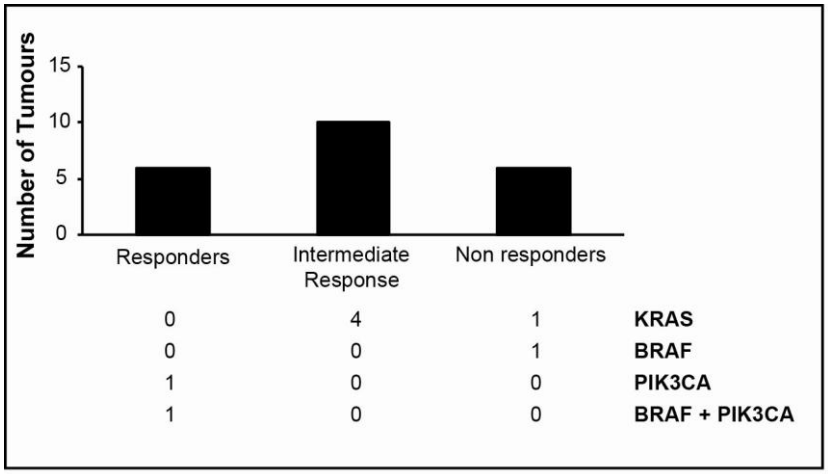


Figure 5.5: Tumour distribution based on sensitivity to AZD6244 and mutational status of *KRAS*, *BRAF* and *PIK3CA*. Figure shows that mutational status of KRAS, BRAF and PIK3K does not correlate with previously established response criteria.

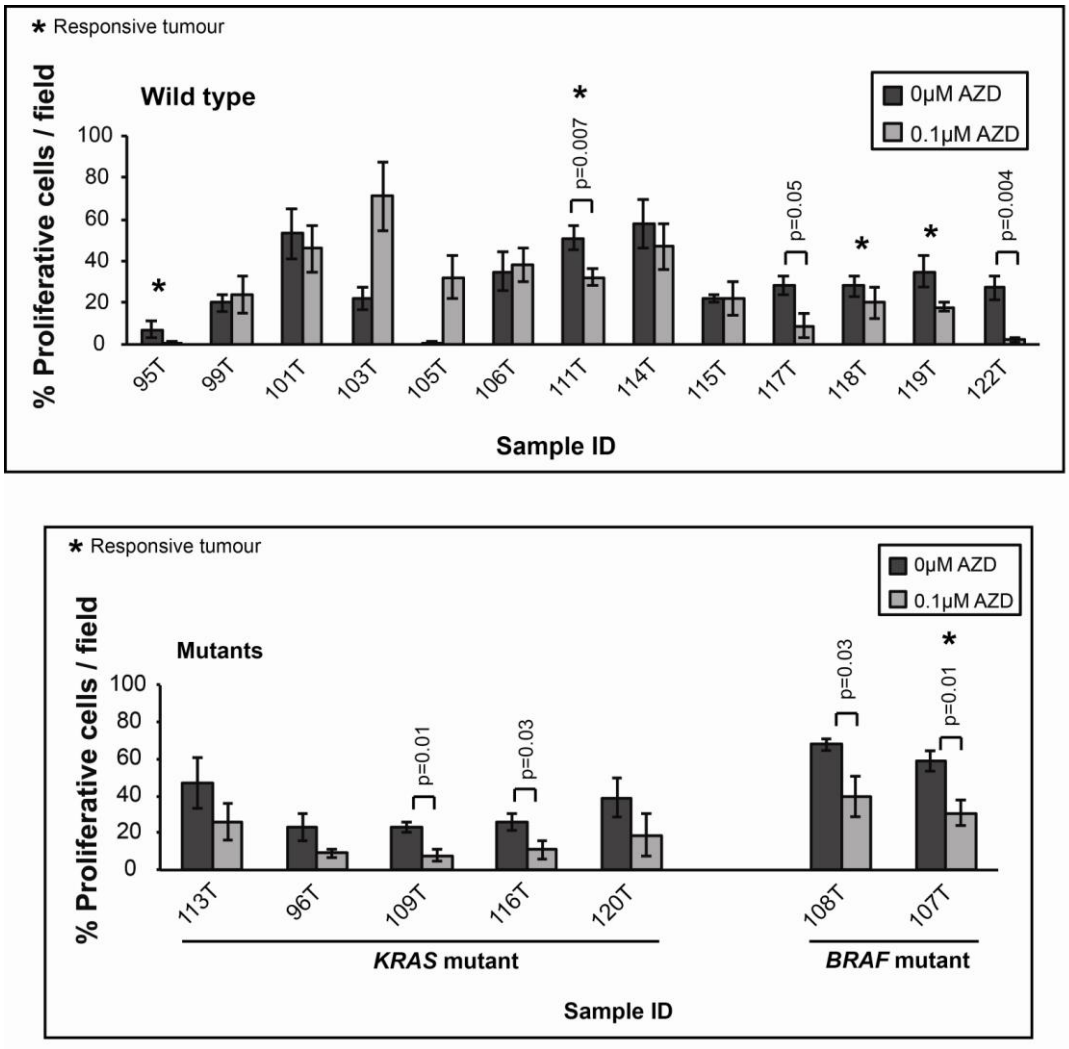


Figure 5.6: Effects of low doses of AZD6244 on proliferation in wild type and *KRAS* and *BRAF* mutant tumours. Immunohistochemical analysis of proliferation marker Ki-67 was performed on tumours treated with 0 and 0.1μM AZD6244 as described in Figure 5.3. Data are the average of 5 fields of view +/- SE. One tail Student's t test was used to calculate p values. Only values of p≤5 are shown. All mutant tumours present inhibition of proliferation in response to AZD6244 and in 4/5 tumours this effect has reached 2-fold. In wild type tumours the effects of this agent are variable and 2-fold inhibition of proliferation was only observed in 2 tumours.

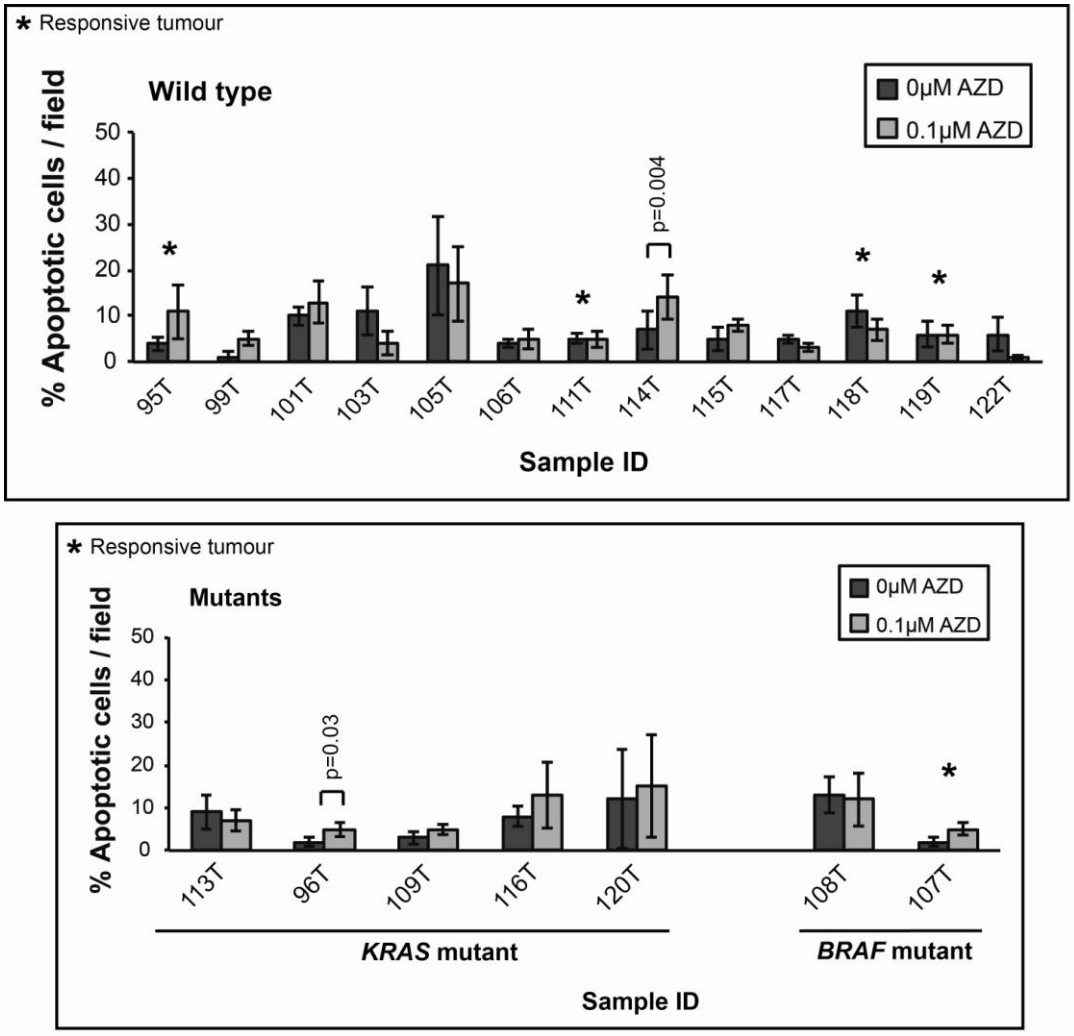


Figure 5.7: Effects of low doses of AZD6244 on apoptosis in wild type and *KRAS* and *BRAF* mutant tumours. Immunohistochemical analysis of apoptosis marker active caspase-3 was performed on tumours treated with 0 and 0.1μM AZD6244 as described in Figure 5.3. Data is the average of 5 fields of view +/- SE. One tail Student's t test was used to calculate p values. Only values of p≤5 are shown. The majority of mutant tumours show induction of apoptosis in response to AZD6244 though this effect did not reach 2-fold in any of the samples. In wild type tumours effect on apoptosis is variable, and only 3 tumours show a 2-fold increase in apoptosis in response to this agent.

Surprisingly, in this model system the presence of *PIK3CA* mutation seems to increase sensitivity to treatment with AZD6244. Tumours carrying this mutation presented a dose-dependent inhibition in proliferation and induction of apoptosis (Figures 5.3 and 5.4). However, the number of samples with this mutation is too small to draw any firm conclusions. Interestingly, increased sensitivity to AZD6244 was also observed in one tumour harbouring both *BRAF* and *PIK3CA* mutations, but again sample size is too small to establish a correlation between genotype and phenotypic effects.

In conclusion, the above data would suggest that the presence of a mutation in *BRAF* or *KRAS* may predict a response to low dose AZD6244.

5.2 - Other markers of response to treatment with AZD6244

In addition to mutational status, other factors have been associated with sensitivity to AZD6244. For example, reports suggest that cells addicted to the MAPK pathway are more likely to respond to treatment with AZD6244 (*234*, *235*). Therefore, I hypothesised that the level of ERK1/2 activation at T0 could be an indicator of the changes observed in proliferation and apoptosis in response to AZD6244. However, comparison of p-ERK1/2 levels between T0 samples showed significant variability in the levels of this marker in all of previously established response groups. Therefore, no direct association between levels of p-ERK1/2 expression and response to treatment with AZD6244 was established (Figure 5.8).

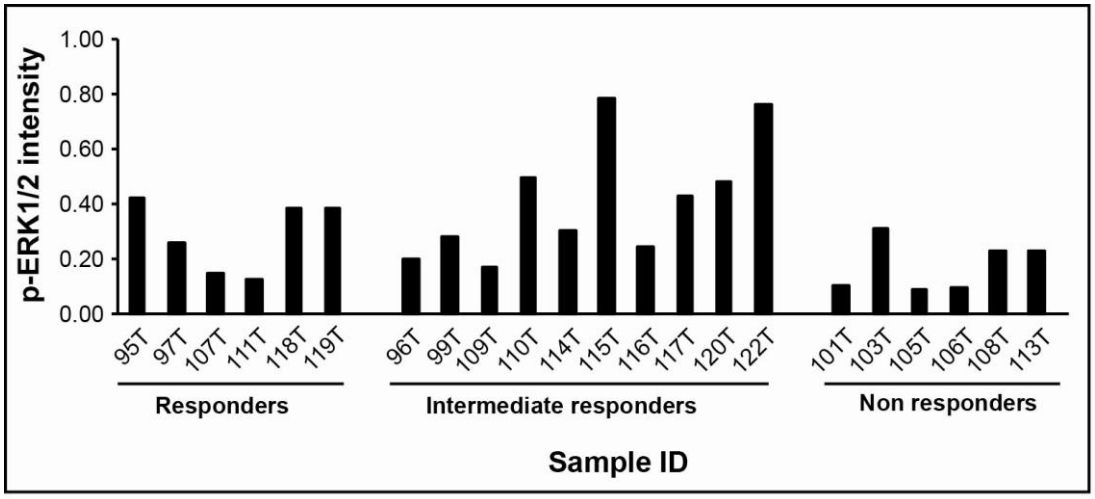


Figure 5.8: Basal ERK1/2 activity does not correlate with phenotypic response to AZD6244. Graph shows the expression of p-ERK1/2 at T0 obtained by western blot analysis as described in Figure 5.1. All T0 samples were examined in parallel in a total of 2 gels. Expression of this marker is variable between tumours displaying the same phenotypic response pattern indicating that there is no association between the expression of p-ERK1/2 at T0 and response to AZD6244. One tail Student's t test was used to calculate the p value between responders and non responders. p=0.04.

Balmanno *et al* previously suggested that high basal levels of p-Akt and Cyclin D1 can be indicators of resistance to AZD6244, in cell lines (*114*). Therefore, I analysed the expression of these markers at T0 in all tumour groups. Western blot analysis showed that there was no correlation between basal levels of Cyclin D1 and tumour response to AZD6244 (Figure 5.9). However, I did find that basal Akt activity was higher in the intermediate responding and non responding tumours (Figure 5.9).

Taken together, these data indicate that markers of sensitivity currently identified in cell line and xenograft models are not reflected in this explant model of colorectal cancer. Using a candidate approach and Reverse Phase Protein Arrays I set out to evaluate the expression of a variety of molecular markers, in a subset of responding and non responding tumours, to determine the molecular differences between these two response patterns.

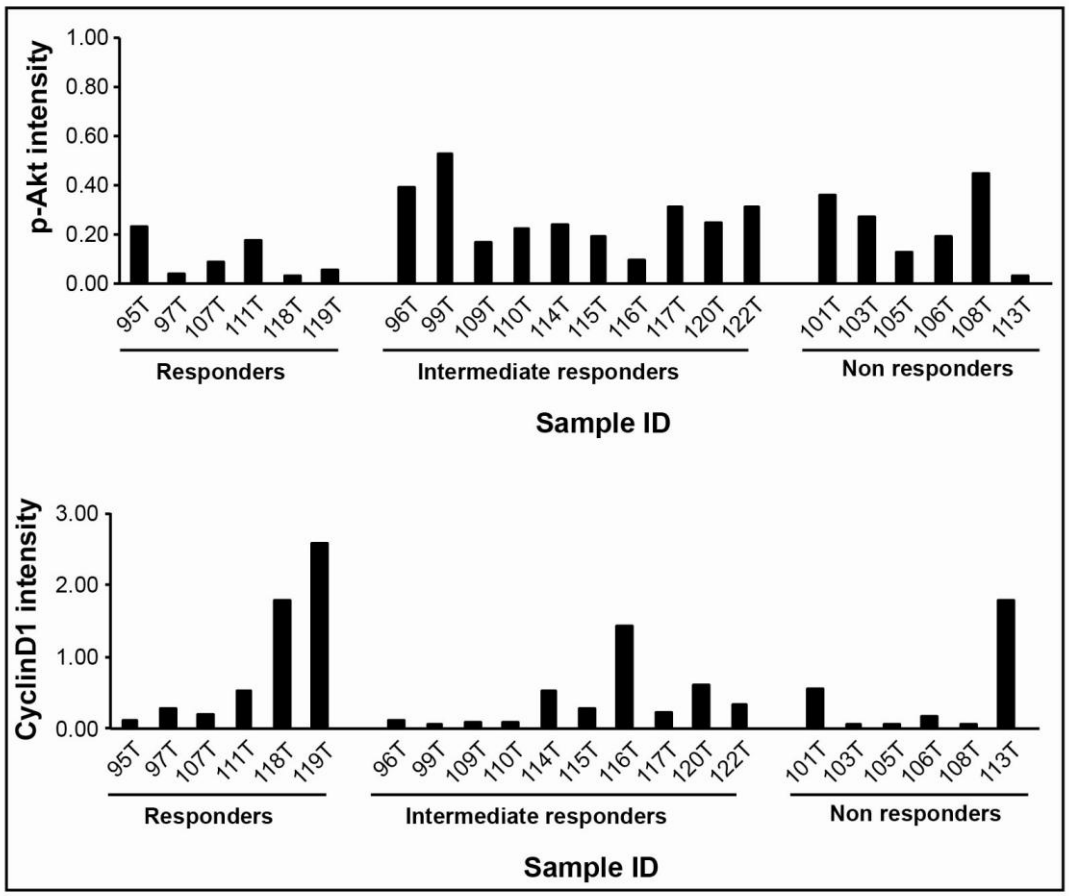


Figure 5.9: Basal activity of Akt and Cyclin D1 does not correlate with phenotypic response to AZD6244. Graph shows the expression of p-Akt and Cyclin D1 at T0 obtained by western blot analysis with antibodies for native and phosphorylated Akt, and Cyclin D1. β-Actin was used as loading control. Protein levels were normalised with β-actin, and phosphorylated Akt was normalised with native protein. All T0 samples were examined in parallel in a total of 2 gels. Data shows that Cyclin D1 levels are variable between tumours displaying the same phenotypic response pattern indicating there is no association between the levels of this protein at T0 and response to AZD6244. One tail Student's t test was used to calculate the p value between responders and non responders. p=0.051. Interestingly, p-Akt levels seem to be increased in tumours classified as intermediate and non responders. One tail Student's t test was used to calculate the p value between responders and non responders (p=0.1).

5.5 – Reverse Phase Protein Analysis

Reverse phase protein arrays (RRPA) are an inexpensive, sensitive, high-throughput, proteomic technology which allows parallel quantification of protein targets on multiple samples in an array-based format. Researchers at the IGMM have previously used this technique to quantify differential expression of active (e.g. phosphorylated) and parental proteins in human breast and ovary tissue in response to drug treatments. The high throughput of the method together with the fact that relatively small concentrations of proteins are required, make this assay ideal for analysis of my panel of tumours treated with AZD6244.

For this assay, I used samples from three responding tumours and three non-responding tumours as I believed this would be the most likely approach to reveal markers of resistance/response with the resources available. Protein lysates from these samples were spotted onto nitrocellulose coated glass slides. These slides were probed with antibodies to 39 selected proteins. For some of these proteins, antibodies to the native and the phosphorylated form were used, making a total of 64 antibodies. The proteins analysed included proteins involved in the maintenance of basic cellular functions like ubiquitination, cell cycle control, proliferation, signal transduction, DNA repair and stress response and apoptosis. See Table 5.1 for details.

RPPA analysis was performed as an external service in the Edinburgh Cancer Research Centre.

Three proteins (p-ERK1/2, p-Akt and Cyclin D1) that were analysed by RPPA had previously been analysed by western blot analysis. Therefore, firstly I compared the expression of these proteins in T0 samples obtained by western blot analysis and using the RPPA assay.

Cell cycle	Proliferation & Survival	Apoptosis	Stress response & DNA damage	Inflammatory response	Wnt pathway members	Tyrosine Kinases	ERK1/2 targets	Transcription factors	Tumour suppressor	Other functions
AMPkα	mTOR	Bad	HSP27 (HSPB1)	IKK	β-Catenin	JAK1	MNK1	Smad	PTEN	β-Actin
Erb-2/HER2/EGFR	p70	Caspase-3	p38	NFκB	c-myc	Src	MSK1	c-jun	TSC-2/Tuberin	β-Tubulin
Cyclin D1	Akt	PARP	EGFR		GSK-3-β	TYK2		Prohibitin	p53	Ubiquitin
Erb-1		SAPk/JNK	ATM/ATR							
p21			p90							
PKC										
LKB1										
Erb-3										

Table 5.1: Markers analysed using RPPA analysis and their cellular functions. Table provides a list of the proteins analysed using RPPA's and which cellular functions they are responsible for or are involved with.

I found that p-ERK1/2 expression at T0 was remarkably similar between assays, and that there was good correlation between the p-Akt levels detected by western blot analysis and with the RPPA (Figure 5.10). However, some discrepancy between assays was observed in the expression of Cyclin D1. As I previously mentioned, the Cyclin D1 signal in western blot assays was very poor, and in fact, intensity achieved in the RPPA was much higher. This suggests that levels of Cyclin D1 obtained by RPPA analysis might be more accurate possibly due to the use of a serial protein dilutions or a better antibody (Figure 5.10). Next, I compared results obtained with RPPA and western blot analysis for AZD6244-mediated inhibition of p-ERK1/2. Again, I found that patterns of p-ERK1/2 inhibition obtained by western blot analysis strongly correlated with those obtained with the RPPA (Figure 5.11). These data were very encouraging as it supported the validity of my previous findings with p-ERK1/2, and supported the use of RPPA. However, when the rest of the RPPA data was analysed, I found that I could not relate any protein expression or phosphorylation status changes with response to AZD6244. A summary of these data is presented in table 5.2.

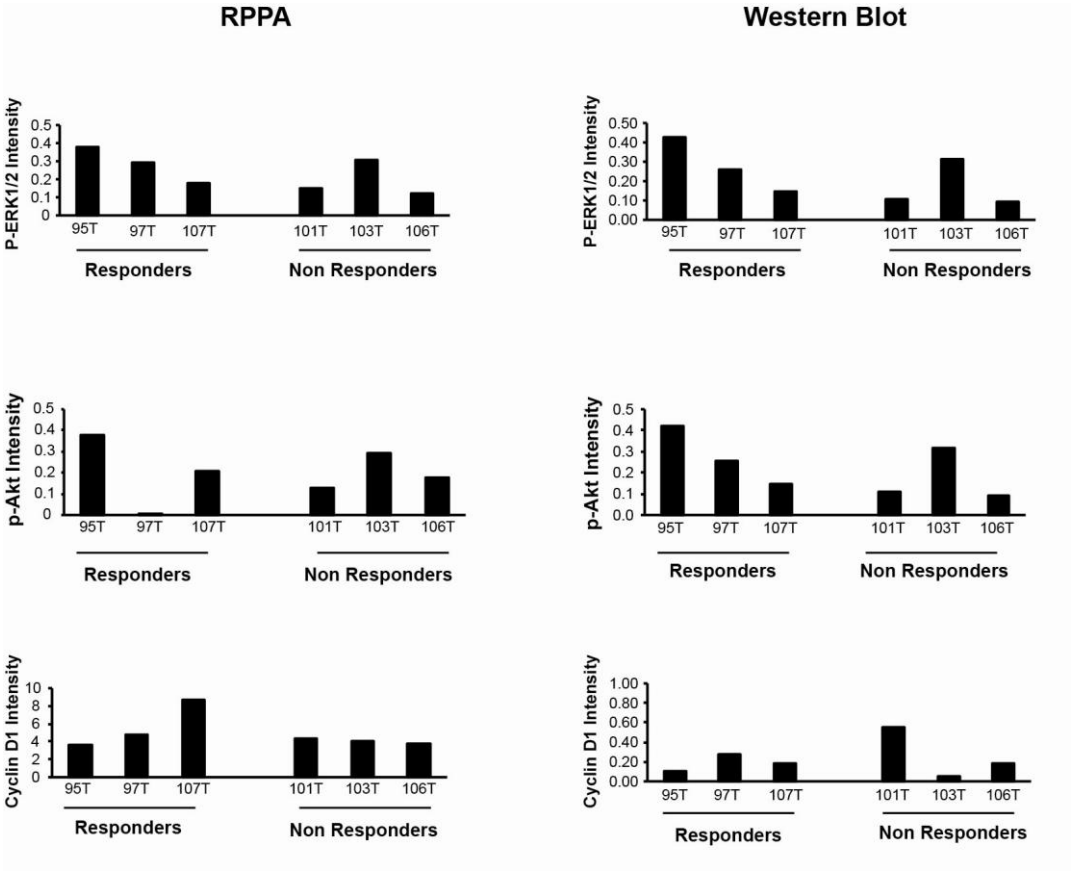


Figure 5.10: Western blot analysis data presents similarities with RPPA data. Western blot analysis was performed on whole cell lysates of two subsets of responding and non responding tumours, as described in Figures 5.1 and 5.9. RPPA analysis was performed on the same lysates. Graphs show that good correlation between two assays was obtained for p-ERK1/2 and p-Akt levels, but not Cyclin D1.

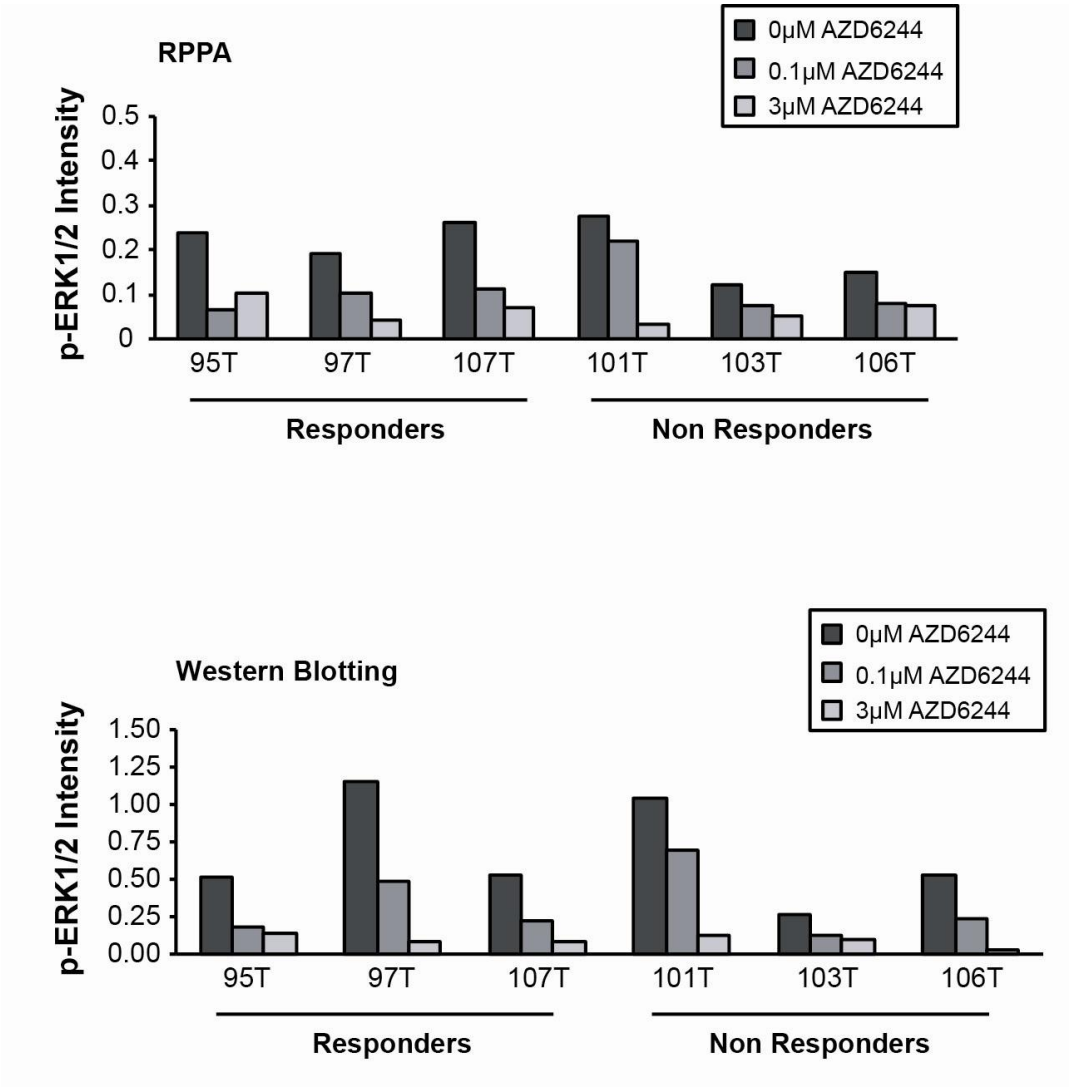


Figure 5.11: Western blot and RPPA analysis of AZD6244-induced p-ERK1/2 inhibition. Western blot analysis was performed on whole cell lysates of two subsets of responding and non responding tumours treated with 0-3μM AZD6244, as described in Figure 5.1. RPPM analysis was performed on the same cell lysates. Graphs show strong correlation between data obtained with the two assays.

		p-AMPK α	p-ErbB-2 / Her2 / EGFR	p-Cyclin D1	p21	PKC	ErbB-1 /EGFR	p-ErbB-3 / Her3 / EGFR	p-mTOR	p-p70	Bad	PARP
95T	T0	5.0	2.1	2.3	40.4	0.9	2.2	8.7	0.5	2.1	0.2	5.0
	0μM	1.8	1.9	2.7	40.0	1.5	2.5	8.0	0.4	1.6	0.4	5.6
	0.1μM	5.0	2.2	3.9	38.0	1.4	1.2	6.2	0.4	2.4	0.5	4.9
	3μM	2.0	2.1	4.8	31.1	1.2	1.7	9.0	0.6	1.0	0.0	3.8
97T	T0	2.0	1.6	5.3	48.7	0.9	2.6	7.2	0.4	9.4	0.8	8.2
	0μM	2.6	1.4	4.0	40.7	0.6	3.1	5.1	0.3	5.0	0.0	6.9
	0.1μM	1.1	2.6	2.7	46.0	1.1	3.4	6.6	0.4	3.1	0.7	7.8
	3μM	1.8	2.3	4.4	43.4	1.5	2.8	6.8	0.4	6.1	0.0	7.3
107T	T0	13.4	2.0	2.7	49.7	1.9	6.0	5.1	0.4	5.7	0.6	11.7
	0μM	7.6	2.5	3.7	55.0	1.8	6.2	4.4	0.4	6.9	0.0	9.9
	0.1μM	6.3	2.5	2.5	37.2	0.9	3.3	6.0	0.5	2.3	0.5	12.5
	3μM	4.5	4.8	5.4	65.3	2.1	4.7	5.0	0.4	8.1	0.4	19.0
101T	T0	2.7	3.1	5.0	45.7	1.6	3.3	5.4	0.4	8.2	0.5	11.6
	0μM	1.6	1.7	4.3	43.1	1.3	1.4	5.2	0.4	9.0	0.6	10.7
	0.1μM	4.4	2.5	8.1	45.8	0.8	2.3	5.3	0.5	5.9	1.8	10.7
	3μM	1.9	2.1	4.2	50.8	1.4	3.3	5.5	0.4	7.5	0.4	13.9
103T	T0	7.3	2.7	4.1	53.4	1.3	3.8	7.0	0.5	2.1	0.5	8.2
	0μM	4.7	3.0	4.4	46.2	2.0	3.8	5.5	0.5	4.3	0.5	7.1
	0.1μM	7.4	2.0	3.0	43.9	1.6	4.7	4.8	0.5	3.1	1.0	6.8
	3μM	4.3	2.7	7.0	46.2	2.0	3.4	4.4	0.3	8.2	0.8	8.3
106T	T0	7.1	2.9	3.7	48.2	1.0	3.6	6.2	0.4	3.2	0.2	13.1
	0μM	2.7	2.5	2.6	39.0	1.0	4.6	5.1	0.5	0.8	0.1	9.3
	0.1μM	9.8	0.4	4.9	45.3	2.4	2.2	7.3	0.4	6.7	2.9	12.0
	3μM	7.7	0.7	8.7	36.4	2.3	3.2	6.2	0.4	2.6	1.1	7.9

		p-SAP / JNK	p-HSP27	p-p38	p-EGFR	ATM / ATR	p-IKKα	NFκB	β-Catenin	c-Myc	GSK-3-β	JAK1
95T	T0	4.2	220.2	1.6	0.0	2.4	10.8	0.2	21.2	3.7	11.6	5.8
	0μM	1.0	132.5	1.0	0.0	1.7	10.8	1.3	15.2	4.2	15.4	7.4
	0.1μM	3.9	4.6	0.7	7.5	2.7	12.0	1.1	37.8	3.3	12.5	7.9
	3μM	2.8	7.0	0.2	7.1	1.6	8.3	0.8	6.3	3.1	13.4	6.8
97T	T0	5.2	6.2	0.7	8.7	2.1	14.5	1.0	40.9	4.8	28.1	9.7
	0μM	3.0	4.6	1.0	9.5	1.6	13.1	1.2	26.9	4.2	27.3	6.1
	0.1μM	4.0	3.3	0.7	11.9	1.9	10.6	1.2	25.3	4.5	25.5	8.2
	3μM	4.1	3.9	0.5	9.4	2.2	13.1	1.0	32.6	5.6	30.1	7.0
107T	T0	8.7	3.8	0.9	10.6	2.1	16.7	0.9	49.6	6.4	33.7	13.3
	0μM	8.7	3.0	1.0	13.0	2.0	14.9	1.2	41.9	6.8	29.9	11.4
	0.1μM	3.9	1.1	0.9	15.4	2.7	11.0	1.4	26.1	4.2	16.3	6.6
	3μM	7.1	4.1	0.7	14.0	2.4	19.0	0.9	37.8	7.5	43.6	11.9
101T	T0	5.9	5.1	0.3	10.2	2.0	14.2	1.5	26.5	5.7	29.9	11.4
	0μM	3.6	4.5	0.2	10.0	2.0	13.4	1.7	34.9	4.6	30.3	6.1
	0.1μM	4.8	4.4	0.8	13.3	2.3	16.4	1.3	40.6	6.2	32.0	7.7
	3μM	3.0	3.5	0.7	11.5	1.7	17.4	1.1	32.4	6.7	39.9	9.6
103T	T0	4.4	3.5	0.9	11.1	2.2	14.2	1.6	52.9	5.4	21.0	10.0
	0μM	4.2	2.3	0.9	16.1	2.3	11.8	0.9	53.9	5.5	19.5	8.4
	0.1μM	4.6	3.2	1.7	11.7	2.0	10.8	1.5	49.0	4.8	15.8	7.5
	3μM	4.7	5.1	0.9	10.8	1.8	14.3	0.8	74.5	5.0	27.5	8.3
106T	T0	4.7	11.4	1.0	8.3	2.1	12.8	0.6	42.3	7.0	21.1	10.5
	0μM	3.8	2.6	1.6	10.0	1.6	9.2	1.2	39.6	5.1	12.3	6.4
	0.1μM	1.9	6.8	0.8	10.5	1.9	11.5	1.6	59.4	6.3	29.6	11.3
	3μM	0.6	8.1	0.8	7.7	4.9	9.6	1.5	31.9	8.4	20.8	10.1

		p-Src	p-TYK2	MNK-1	MSK-1	p-Smad2/3	c-Jun	Prohibitin	p-PTEN	p-TCS-2	p53	Ubiquitin
95T	T0	2.0	2.0	26.2	1.9	1.1	0.7	9.2	1.4	0.1	2.6	0.1
	0μM	4.2	2.3	26.3	1.5	2.0	0.9	12.3	1.3	2.0	2.4	0.0
	0.1μM	2.6	2.9	27.5	2.1	1.9	0.5	14.9	0.9	2.0	3.7	1.0
	3μM	3.8	1.9	27.5	1.8	3.3	0.2	6.3	1.4	2.1	4.1	0.5
97T	T0	5.3	1.7	36.3	3.4	3.0	1.2	12.0	1.6	3.6	4.0	0.7
	0μM	4.1	1.8	26.9	3.0	3.7	0.7	10.9	1.3	3.4	4.3	0.4
	0.1μM	4.9	2.5	27.5	1.1	2.6	1.8	15.2	0.9	3.1	3.5	0.8
	3μM	3.0	1.6	32.0	2.5	3.5	1.4	10.6	1.3	2.7	4.9	0.8
107T	T0	5.6	1.7	38.6	2.4	5.9	1.1	20.0	1.4	3.8	6.2	1.7
	0μM	6.6	3.8	36.2	3.4	5.3	2.0	21.3	1.3	4.2	5.3	1.0
	0.1μM	3.3	3.0	24.0	1.7	2.2	1.5	16.3	1.6	3.9	4.3	2.9
	3μM	4.9	2.2	41.8	4.4	7.1	1.5	26.1	1.9	4.8	4.7	1.8
101T	T0	4.7	3.0	32.3	0.7	4.3	0.8	15.9	1.5	2.5	4.0	0.6
	0μM	4.7	3.2	27.9	2.3	4.3	1.6	19.2	1.8	3.5	4.7	0.7
	0.1μM	4.2	2.9	32.3	3.6	4.4	1.3	22.8	2.7	2.4	4.7	0.9
	3μM	5.7	2.2	36.5	3.7	5.0	1.9	18.3	2.1	3.9	5.3	1.4
103T	T0	3.6	1.5	40.3	1.7	3.6	1.8	13.6	1.4	2.3	6.1	1.7
	0μM	3.6	2.3	33.4	2.4	4.5	1.9	14.8	1.8	4.8	6.1	1.7
	0.1μM	3.2	2.1	28.9	2.1	3.5	2.1	13.6	1.0	2.7	5.9	1.2
	3μM	4.3	1.9	31.4	2.4	4.2	1.8	15.8	2.5	3.3	7.4	1.1
106T	T0	3.6	1.9	35.9	0.6	4.0	1.6	14.3	0.9	2.1	3.6	0.9
	0μM	4.2	2.5	25.6	1.2	3.0	1.3	16.6	1.0	2.0	3.0	1.2
	0.1μM	2.3	2.6	37.3	2.0	0.7	1.0	18.1	1.6	0.0	5.5	1.1
	3μM	3.5	1.6	34.0	2.5	0.0	1.2	20.9	1.9	0.0	4.7	0.9

Table 5.2: Summary of RPPA results. Table shows the levels of an array of markers evaluated using RPPA analysis in a subset of responding and non responding tumours treated with 0, 0.1 and 3μM AZD6244 for 1h.

RPPA analysis also failed to provide an indication of the effects of AZD6244 on proliferation and apoptosis, especially in responder tumours where a dramatic phenotypic effect was detected by immunohistochemical analysis. Levels of proliferation marker Ki-67 detected with this assay were very low, and therefore, could not be related with the levels previously obtained by immunohistochemical analysis. In the RPPA, effects on apoptosis were evaluated using antibodies for pro-caspase-3 while by immunohistochemistry it was the levels of active caspase-3 that were investigated. Therefore, I could not compare apoptotic response data obtained with the two assays.

One of the limitations of this particular assay was the fact that a relatively small number of proteins involved in the MAPK pathway could be investigated, and only two ERK1/2 targets were analysed. The remaining proteins analysed are involved in more general cellular functions or are proteins typically activated in cancer, but that might not necessarily be affected by MEK1/2 inhibition with AZD6244.

5.6 – Response to AZD6244 is not correlated with histopathological criteria

It is widely known that patients presenting tumours of the same Dukes or TNM stages present different outcomes and respond differently to the same therapeutic regimes. Therefore, I compared the histopathological data of the patients recruited for this study to determine if there is a correlation between the response to AZD6244 and tumour's stage or other pathological properties.

A summary of the histopathological data can be found in Table 5.3. I found that the tumour stage, either Dukes or TNM, distribution was similar in all response groups. Histological properties and tumour differentiation were also the same in all three groups. I then looked at the patient's age at diagnosis and found that the spectrum of ages was very similar between response groups. These observations further contribute to the notion that response to therapy involves a variety of mechanisms that cannot be predicted based on tumour morphology only, and that comprehensive molecular characterisation of the disease as well as individual tumours are needed in order to accurately predict prognosis and therapeutic outcome.

	Sample ID	Gender	Age at Diagnosis	Site	Differentiation	Histology	Duke's stage	T stage	N stage	M stage
Responders	**95T**	F	59	Rectosigmoid	Moderate	Standard	B	T3	N0	M0
	97T	M	42							
	107T	F	61	Caecum	Moderate	Standard	C	T4	N1	M0
	118T	M	78	sigmoid colon	Moderate	Standard	A	T2	N0	M0
	119T									
	111T	M	74	Sigmoid colon	Moderate	Standard	B	T3	N0	M2
Non Responders	**101T**	M	63	Caecum	Moderate	Standard	B	T3	N0	M0
	103T	F	39	Rectum	Moderate	Standard	C	T2	N1	M0
	105T	M	47	Sigmoid colon	Moderate	Standard	C	T3	N1	M0
	106T	M	69	Rectum	Moderate	Standard	C	T3	N2	M0
	108T	F	66	Caecum	Moderate	Mucinous	C	T3	N1	M0
	113T									
	121T	F	76							M0
Intermediate Responders	**96T**			Rectum	Moderate	Standard	C	T4	N1	M0
	99T	M	79	Caecum	Moderate	Standard	B	T3	N0	M0
	109T	M	71	Ascending/hepatic/ right colon	Moderate	Standard	C	T3	N2	M0
	110T	F	80	Rectosigmoid	Moderate	Standard	B	T2	N0	M2
	114T									
	115T	M	61	sigmoid colon	Moderate	standard	C	T2	N1	M0
	116T	F	74							
	117T	M	63	Rectum	Moderate	Standard	C	T3	N1	M0
	120T	M	73	Rectum	Moderate	Standard	C	T3	N2	M0
	122T	M	69							

Table 5.3: Histopathological data of patient tumours treated with AZD6244. Table shows a summary of the histopathological data of all tumours treated with AZD6244. Data was obtained from the Pathology department at the Western General Hospital.

Discussion:

The frequency of *KRAS* mutations reported in the literature is 40-50%. However, mutational rates vary slightly with geographic region (*98, 236*). Analysis of *KRAS* mutational status in my panel of tumours revealed that 27% were *KRAS* mutant. Interestingly, the same rate of *KRAS* mutations was found in patients admitted for surgery in Scotland, between 1997 and 1999 (*237*).

Contrary to what was expected, tumours carrying *KRAS* or *BRAF* mutations did not present increased basal p-ERK1/2 activity when compared to wild type tumours. One possible explanation is the fact that pathway activation might have been affected during the period between tumour resection and arrival to the lab. As mutant tumours are thought to be addicted to this pathway, this effect would be more dramatic in these tumours then in wild type tumours, resulting in similar basal levels of p-ERK1/2 between samples. The concept of pathway addiction was demonstrated in cell lines (*234, 235*) bringing up the possibility that such observations result from factors inherent to the study model. In an *in vivo* environment where tumour cells are regulated by a variety of factors, pathway addiction and increased levels of p-ERK1/2 might not occur.

I also found that the mutational status of *KRAS* and *BRAF* did not correlate with phenotypic response to 3μM AZD6244. Therefore, as I categorised responder tumours as those that responded at both doses (to remove potential variability in proliferation / apoptosis between tumour fragments) there appeared to be no correlation between KRAS and BRAF mutational status and phenotypic response. However, there seemed to be a correlation between the presence of these mutations and response to 0.1μM of this agent. These data may suggest that with 0.1μM

AZD6244 the response is more specific than with 3μM, or that at higher doses response observed might have resulted from the inactivation of short term feedback mechanisms that control pathway homeostasis and are regulated by p-ERK1/2 (see Introduction). Inhibition of p-ERK1/2 might have prevented feedback inactivation of KRAS or BRAF forcing these kinases to activate alternative effectors like PI3K or IKK, which in turn promote proliferation and inhibit apoptosis compensating for inactivation of the MAPK pathway. However, more work is needed to examine the correlation between *KRAS* and *BRAF* mutations and response to AZD6244. Data also suggests that wild type and mutant tumours present distinct mechanisms of response to the drug and that sensitivity to AZD6244 might vary with the dose of the agent. If that's so, results supporting or denying the importance of these mutations as markers of response might have been influenced by the drug concentrations used by the authors.

Analysis of other markers of response proposed in the literature (*114*) also revealed no correlation between the basal levels of p-Akt and Cyclin D1, and sensitivity to AZD6244 in this study model.

RPPA analysis of p-ERK1/2 and p-Akt levels showed strong correlation with previously performed western blots analysis of the same proteins. Good correlation between the two assays was also found for AZD6244-induced inhibition of p-ERK1/2 in responder and non responder tumours. This indicated that RPPA's are a reliable and robust method of analysis that could be used to detect variations in the levels of a variety of proteins, and establish a molecular signature of response to AZD6244. However, RPPA data revealed no correlation between the levels of other proteins analysed or phosphorylation status changes, and phenotypic response. One

of the shortfalls of the RPPA is that, due to the nature of the assay, antibodies require validation prior to use to assure that the expected sensitivity is achieved in every assay. Antibody validation is performed by comparing results obtained by RPPA and western blot analysis. However, due to low sensitivity of the assay, changes in some proteins cannot be detected by western blot analysis which limits the range of antibodies to be used in RPPA assays. For this reason the number of proteins that I was able to investigate was limited, and it included only a few proteins directly involved in the ERK1/2 pathway.

Higher throughput methods are needed to investigate a wider range of proteins and detect subtle molecular changes in response to AZD6244. In a recently published paper, Dry *et al* (*238*) revealed an 18-gene signature that allows for the measurement of MEK1/2 activity independently of genotype, using RT-qPCR assays. However, this signature was identified in cancer cell lines. Though it is fundamental to test the validity of this 18-gene signature in human colorectal tumours treated with AZD6244 this could not be performed with my panel of samples. The small size of the tumours limited the choice of analysis methods, and therefore RNA samples could not be obtained from the tumours. An alternative might be to extract RNA from fixed tissue.

Taken together the data presented here indicate that even though acute treatment with AZD6244 can induce a strong phenotypic response in human colorectal tumours, the mechanisms behind it are still largely unknown due to the lack of correlation between tumour properties and sensitivity to the drug. Tumours with mutations in *KRAS* or *BRAF* did not present increased ERK1/2 activity as initially expected, and AZD6244-induced p-ERK1/2 inhibition was equivalent in

mutant and wild type tumours. This offers an explanation to why the mutational status of these proteins might not be an indicator of sensitivity to this agent. Expression of markers identified in cell lines also failed to correlate with tumour response to AZD6244 suggesting that tumour properties not found in cell lines, like the presence of stromal cells, extracellular matrix and tumour microenvironment, also have a role to play in therapeutic responses. Nevertheless, acute treatment of patient tumours with AZD6244 together with a higher throughput analysis system that allows for a more comprehensive overview of the changes occurring in response to treatment, and validation methods for the confirmation of the results obtained would make a powerful tool to identify markers of sensitivity that are more relevant to the clinic, and to determine which patients are more likely to benefit from this therapeutic option.

Chapter 6: AZD6244 in combination therapy

Introduction:

AZD6244 has shown some promise in clinical trials. However, the best outcome reported so far is disease stabilisation. This agent effectively reduces tumour growth but it does not consistently reduce tumour size (*115*, *116*). AZD6244 has a cytostatic effect that seems to be enhanced when used in combination with agents that target pathways thought to be involved in the resistance to treatment with this drug. One such pathway is the PI3K pathway, which is activated by RAS (*239*) or by mutations in PI3K (*229*). Mutations in other proteins, such as Akt and PTEN, are also frequently found in a variety of solid tumours, and lead to constitutive activation of the PI3K pathway promoting growth, proliferation and survival (*240*). Combinations of AZD6244 with PI3K, Akt or mTOR inhibitors have been proposed for the treatment of cancers of different origins including colorectal tumours, and have so far shown promise *in vitro* and *in vivo* (*119-121*). Furthermore, AstraZeneca has recently announced a Phase I clinical trial to test combinations of AZD6244 and Akt inhibitor MK-2206 (*241*).

Combinations of AZD6244 and currently used therapeutic agents have also been proposed. Clinical trials to assess the efficacy of AZD6244 and Capecitabine for the treatment of advanced pancreatic cancer are currently ongoing (*242*), and AZD6244 has also been shown to increase sensitivity to the chemotherapy agent docetaxel in melanoma cells (*243*).

Work in the host lab has identified a novel mechanism by which aspirin acts against colorectal cancer cells (see section 4.7 in Chapter 4). Interestingly, upon analysis of this mechanism, I realised that many of the pathways known to cause resistance to AZD6244 are targeted by aspirin. For example, high basal levels of

Cyclin D1, p-ERK1/2 and p-Akt are associated with acquired resistance to AZD6244 (*114*) while aspirin is known to induce degradation of Cyclin D1 (*133*) and de-phosphorylation of ERK1/2 and Akt (*244*). Furthermore, aspirin can inhibit the mTOR pathway which will limit the effects of high p-AKT on cell proliferation and apoptosis (*136*). Overexpression of MEKK1 (RAF) has also been associated with resistance to AZD6244 and it has been suggested that the mechanism is through stimulation of IKK (IκB Kinase) and consequently, activation of NF-κB's anti-apoptotic function (*245, 246*). Aspirin has previously been shown to inhibit NF-κB signalling in colon cancer cells, which may allow tumours to overcome resistance to AZD6244 (*133*). Based on these data, I hypothesised that tumour sensitivity to AZD6244 could be enhanced in the presence of aspirin. However, the outcome of targeting both these pathways simultaneously has not yet been reported. Therefore, I set out to determine the combined effects of aspirin and AZD6244 on colorectal cancer cell growth and death.

Results:

6.1 AZD6244 and aspirin present synergistic effect in colorectal cancer cell lines

In order to test this hypothesis, I firstly used a panel of colorectal cancer cell lines and the Chou-Talalay median-effect method, which is the method most frequently described in the literature to investigate the effects of drug combinations on cell growth. This method, which was developed by Ting-Tao Chao and Paul Talalay in 1983, determines if drug combinations are synergistic, antagonistic or additive using relatively few measurements. It can be applied to both hyperbolic and

sigmoidal dose-response curves, regardless of whether the drugs act independently of each other or not (*247*). This method was used to determine whether aspirin and AZD6244 have a synergistic, antagonistic or additive effect when used in combination.

I selected the same cell lines that were used in Chapter 4 (HRT18, RKO and HCT116) as these differ in their *KRAS* and *BRAF* mutational status (See section 4.1). First, I generated growth curves for each cell line to determine the optimal cell density, and the time point at which each of the cell lines achieved exponential growth. This was done by plating the cells at increasing densities in 96-well plates and growing them over a period of 7 days. Cells were harvested daily, fixed and a Sulforhodamine B colourimetric assay was performed to determine cell viability. Plates were read with a spectrophotometer and absorbance values determined at 540nm, were used to generate growth curves for each cell lines (Figure 6.1). I then proceeded to establish the EC_{50} values of each drug in each cell line. Cells were plated at optimal density and allowed to grow until exponential growth was achieved then treated with increasing concentrations of AZD6244 or aspirin for 72h. Again, a sulforhodamine B assay was performed to determine cell viability. Absorbance values were then used to generate dose response curves and calculate EC_{50} values using Prism software.

Based on these data, cells were plated, left until they were growing exponentially then treated with 0-25mM aspirin and 0-135µM AZD6244 individually and in combinations, for a period of 72h. Six experimental repeats were used per assay, and each assay was performed in three independent experiments.

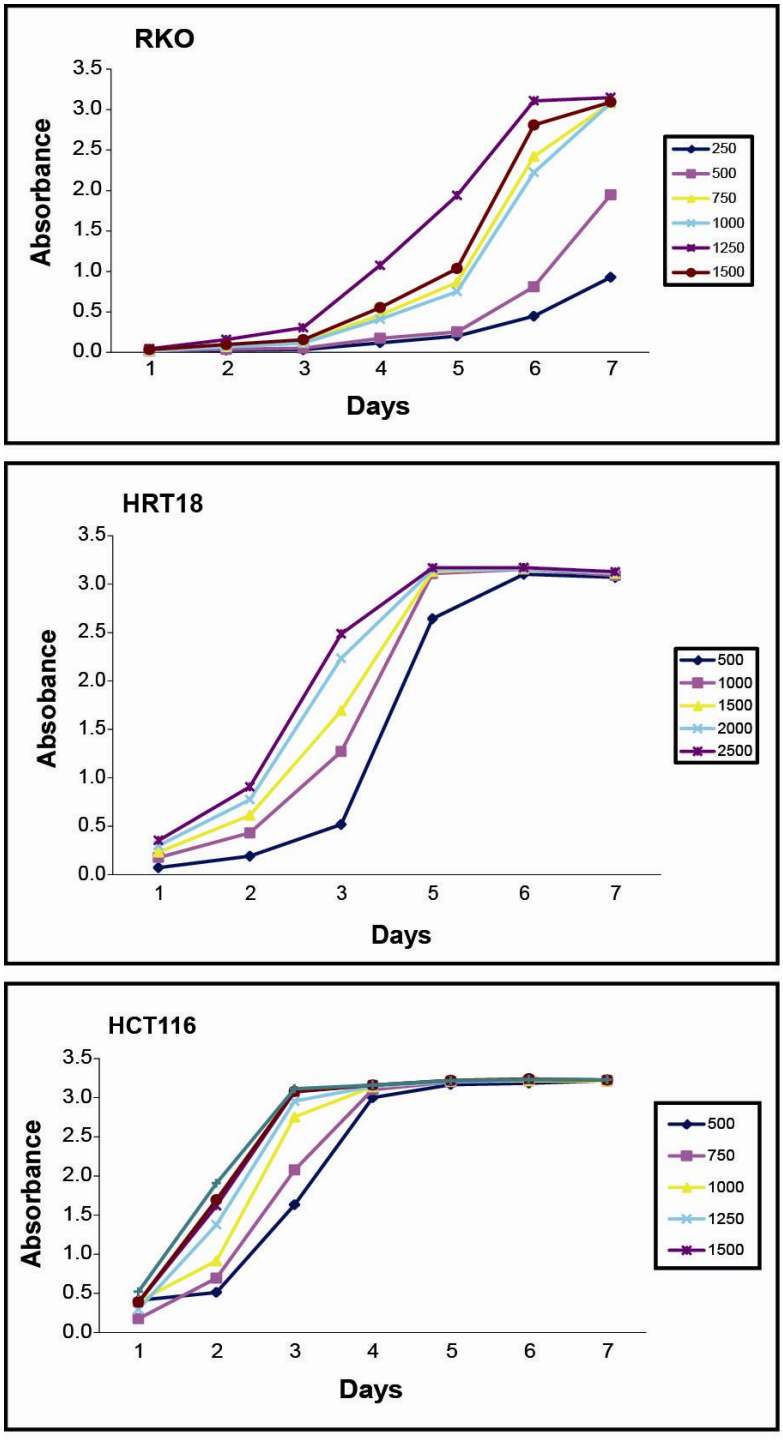


Figure 6.1: Growth curves for RKO, HRT18 and HCT116 cells. Cells were plated at increasing densities (250 to 2500 cells per well) in 96-well plates and grown for up to 7 days. Cells were harvested daily, fixed and cell viability was determined using a Sulforhodamine B assay. Data shows the absorbance values measured each day for each cell line.

Using Compusyn software, I was able to calculate the Combination Index (CI) values for each treatment option, and generate Fraction affected-Combination index (Fa-CI) Plots and isobolograms for each cell line (Figure 6.2). The Combination Index is used to quantify synergism or antagonism between two drugs, CI<1 indicates synergism, CI>1 indicates antagonism, and CI=1 indicates that the drugs have an additive effect (*248*). The fraction affected or Fa is the percentage of cells killed by the drug. Fa-CI Plots are a quick and easy graphic way to determine the nature of each drug combination and how it affected cell survival. Isobolograms also illustrate the nature of each drug combination but in a dose rather than effect orientated fashion, quickly providing an indication of the nature of the effects of each drug. If the combination data points fall on the hypothenuse an additive effect is indicated, if they fall on the lower left or upper right then combinations are synergistic or antagonistic, respectively. Classic isobolograms are generated for constant drug ratios while Normalised isobolograms are used for non constant drug ratios (*248*).

Results show that in HRT18, HCT116 and RKO cell lines, a number of drug combinations tested present CI values far below 1, indicating a synergistic effect (Figure 6.2). This is most apparent when low concentrations of both aspirin and AZD6244 are combined. As non constant drug ratios were used, normalised isobolograms were generated. These show that, in all cell lines tested, the vast majority of AZD6244 and aspirin combinations used fall below the hypothenuse further confirming the synergistic effect of the drug combinations (Figure 6.2). High concentrations of AZD6244 and aspirin used individually or in combination result in dramatic cell death due to high toxicity.

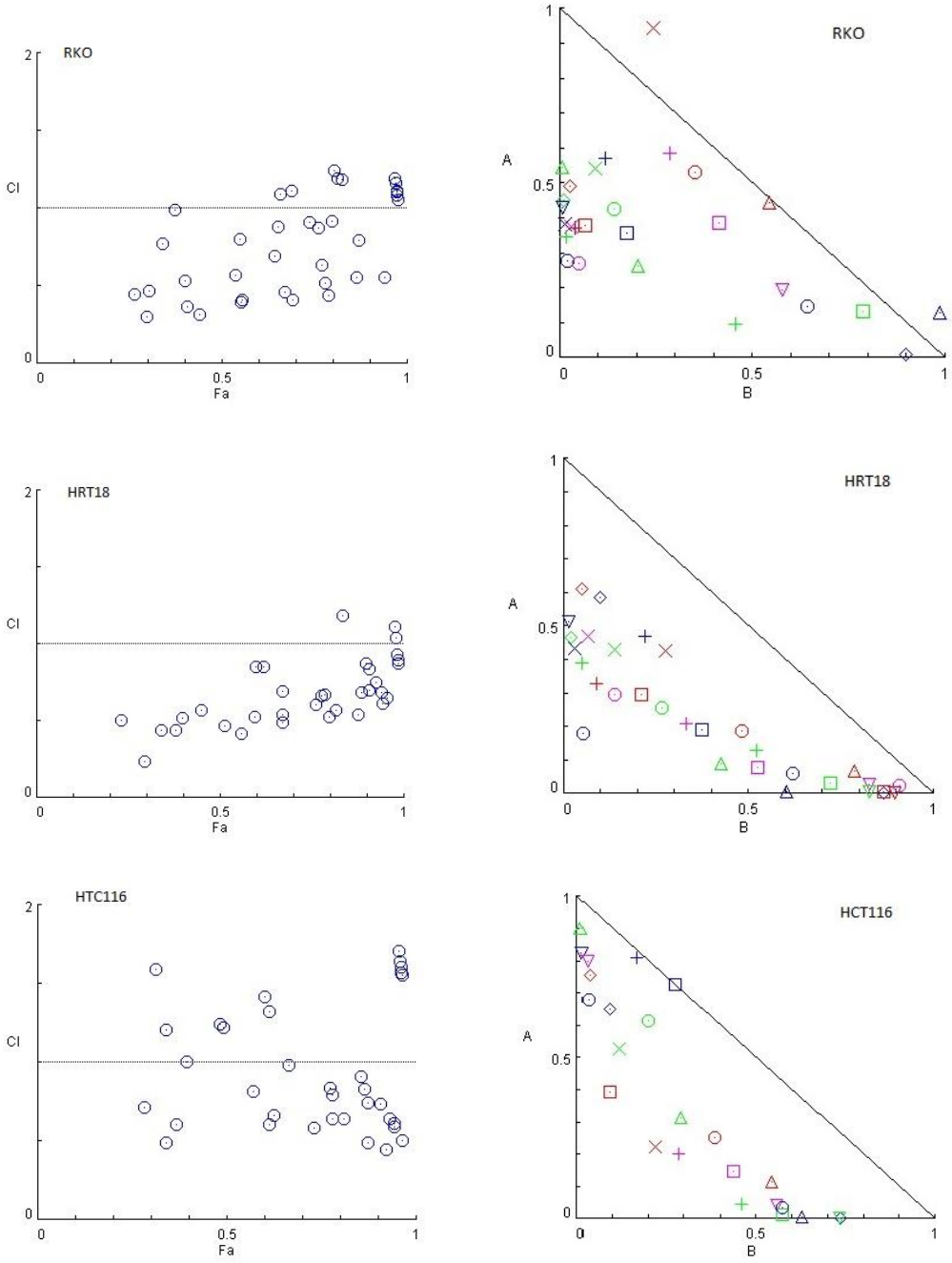


Figure 6.2: Aspirin and AZD6244 have synergistic effect in all cell lines tested. Cells were treated with increasing concentrations of AZD6244 and aspirin, individually and in combination, for 72h. Sulforhodamine B assay was used to determine cell viability at the end of treatment, and Compusyn software was used to generate Fa-CI plots (left), and isobolograms (right) shown above. Fa-CI plots show that the vast majority of drug combinations, shown as dots on the graph, are synergistic (CI<1). These results are further illustrated by the normalised isobolograms where drug combination data points that fall on the lower left hand side or on the hypothenuse correspond to the Fa-CI plot values ≤1.

Combinations of high concentrations of each drug have an additive rather then synergistic effect shown by CI=1. Therefore, no advantage is gained with these combinations when compared to individual effects.

To further illustrate the synergistic effects of combination therapy with aspirin and AZD6244, I compared the levels of cell death caused by one drug individually and combined with increasing concentrations of the second drug. Again I found that in HRT18 and RKO cells the most advantageous combinations are the ones using the lowest doses of each drug (Figure 6.3). For example, in RKO cells treated with 0.7μM AZD6244 effects on cell death were negligible, but in cells treated with the same concentration of AZD6244 combined with 0.05mM aspirin, cell death increased to over 40%. Similarly, HRT18 cells treated with 0.45μM AZD6244 combined with 0.08mM aspirin showed nearly 40% increase in cell death when compared with cells treated with AZD6244 alone. However, in HCT116 cells combination of the two agents is not as advantageous. HCT116 cells are more sensitive to AZD6244 and therefore higher cell death rates are observed even with low concentrations of this agent (Figure 6.3). In all cell lines, treatment with aspirin alone had minimal effect on cell viability.

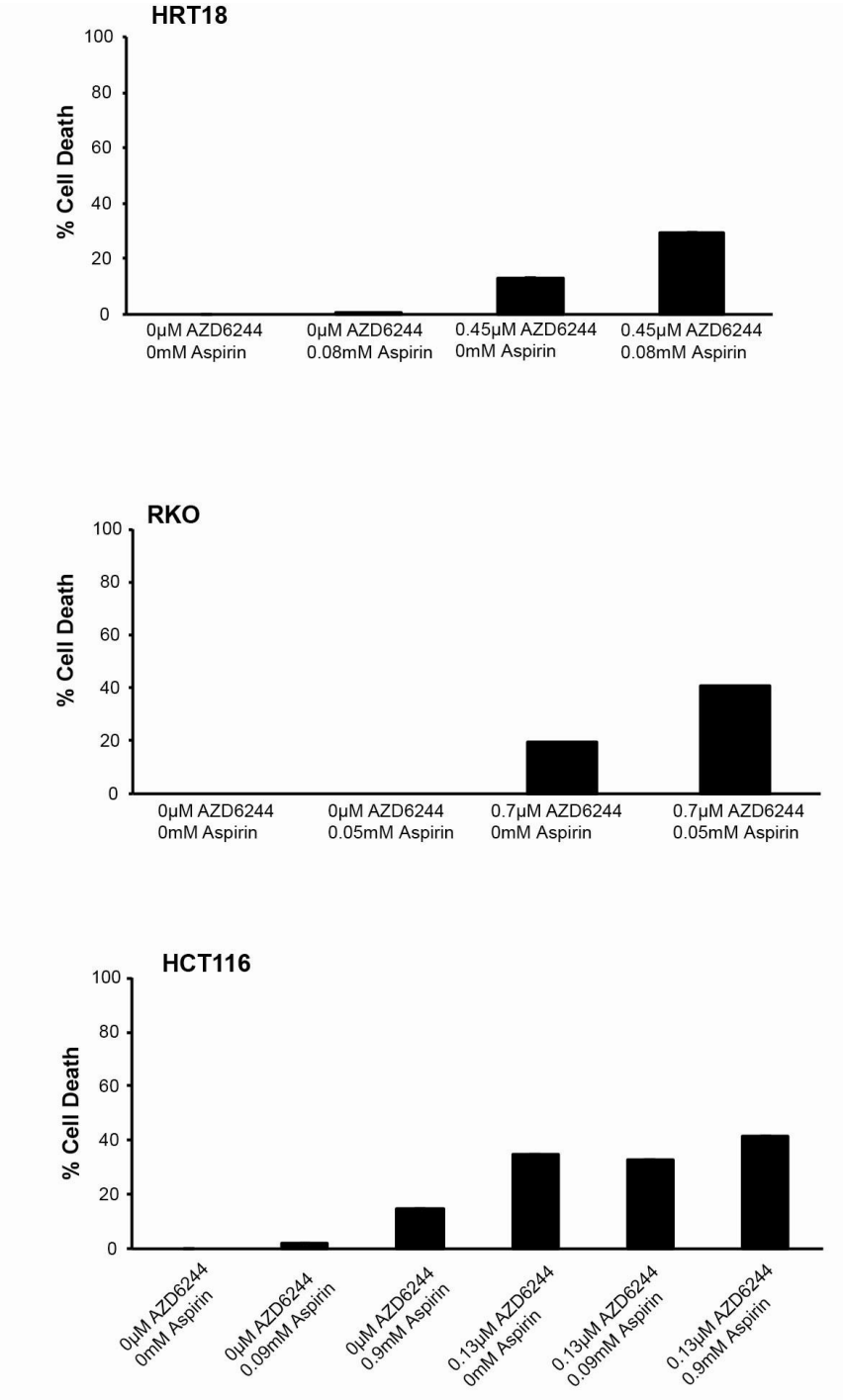


Figure 6.3: Combined effect of AZD6244 and aspirin on cell viability. RKO, HRT18 and HCT116 cells were treated with increasing concentrations of AZD6244 and aspirin individually and in combination as described in Figure 6.2. Data shows the percentage of cell death that occurred as a result of each treatment regime. In HRT18 and RKO cells, treatment with combinations of the two agents results in a 2-fold increase in cell death when compared to AZD6244 alone. In HCT116 this effect is less apparent and higher doses of aspirin are needed in combination with AZD6244 in order to obtain a relatively small increase in cell death.

6.2 AZD6244 and aspirin combinations in tumour explants

Having demonstrated that combinations of aspirin and AZD6244 have a synergistic effect in colorectal cancer cell lines, I proceeded to treat patient tumour explants with AZD6244 and aspirin, individually and in combination. Tumours were processed as in Figure 4.3 and as outlined in Chapter 4 then treated with 0, 0.1 and 3μM AZD6244 alone or in combination with 100μM aspirin, in the presence of 0.03% DMSO. No DMSO was added to samples treated with 100μM aspirin only and respective non treated controls. Data from the samples treated with AZD6244 or aspirin alone are included in the tumour panel outlined in Chapter 4. The number of samples treated with this drug combination was limited by the size of the tumours. However, in total, 8 tumours were treated with combinations of AZD6244 and aspirin.

6.2.1 The presence of low doses of aspirin modifies pharmacodynamic response to AZD6244

Firstly, I examined aspirin effects on AZD6244-mediated inhibition of p-ERK1/2 using western blot analysis, followed by ImageJ quantification as outlined in section 4.3. I found that 100uM aspirin alone had a minimal effect on p-ERK1/2 levels after 1h of treatment in most tumours (Figure 6.4). I also found that in all tumours, the presence of aspirin reduced AZD6244-mediated inhibition of p-ERK1/2. This effect was most apparent when combinations of 0.1μM AZD6244 and 100μM aspirin were used (Figure 6.5). These results would suggest that, in contrast to cell line data, the drugs are having an antagonistic effect, especially at the more specific 0.1uM dose of AZD6244.

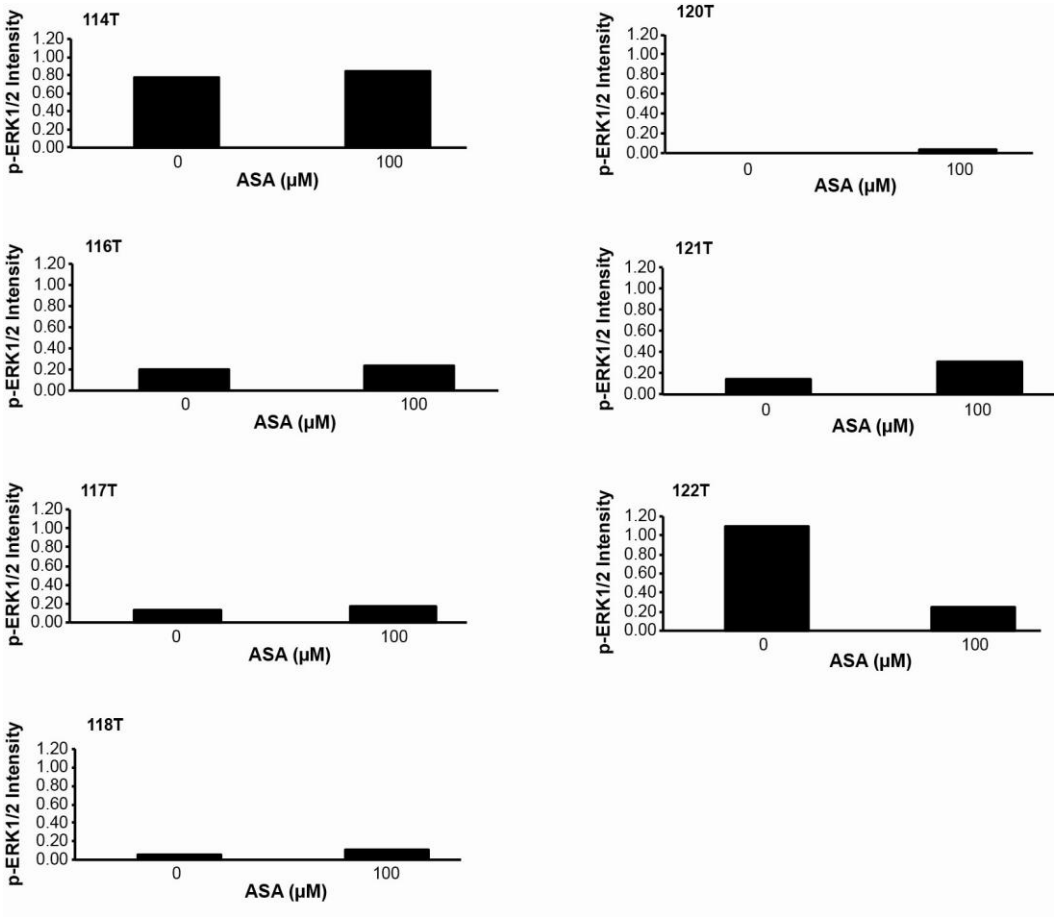


Figure 6.4: Treatment with 100μM aspirin for 1h does not fully inhibit p-ERK1/2 activity. Tumours were treated with 0 and 100μM aspirin for 1h and western blot analysis was performed as described in Figure 4.5. Data indicates that treatment with 100μM aspirin was not sufficient to inhibit ERK1/2 phosphorylation in the vast majority of tumours treated.

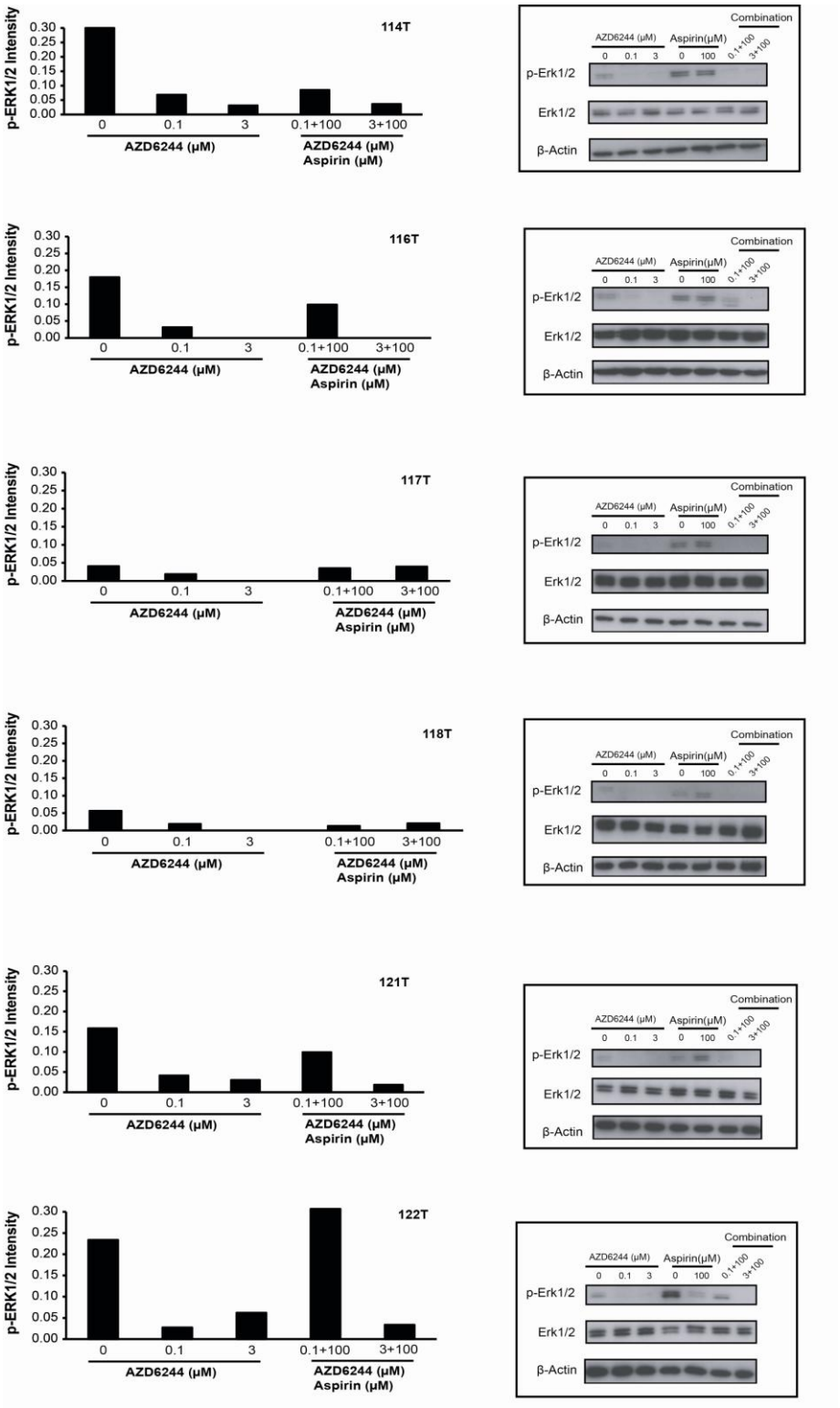


Figure 6.5: Aspirin reduces AZD6244-induced inhibition of p-ERK1/2. Tumours were treated with 0, 0.1 and 3μM AZD6244 alone or in combination with 100μM aspirin for 1h and western blot analysis was performed to determine the effects of the combinations on p-ERK1/2 activity, as described in Figure 4.5. Western blots are shown on the right and respective blot intensity quantification data is shown in graphs on the left.

6.2.2 Aspirin can increase sensitivity to AZD6244 in a subset of tumours

In order to establish if aspirin increased patient tumour sensitivity to AZD6244, despite effects on p-ERK1/2, I quantified levels of proliferation marker Ki-67 and apoptosis marker active caspase-3 in samples treated with AZD6244 individually and in combination with 100μM aspirin (as described in section 4.3).

Using the response criteria previously established (See section 4.4) I found that aspirin can increase tumour sensitivity to AZD6244 in samples that show an intermediate response to treatment with AZD6244 alone (Table 6.1). However, increased sensitivity to AZD6244 by aspirin was only observed in 3 tumours, and even within this group, the extent to which tumours were sensitised varied between samples. In two tumours a dose-dependent inhibition of proliferation and increase of apoptosis was observed in samples treated with combinations of AZD6244 and aspirin. These two tumours were therefore classified as responders when treated with combination regimes but not with AZD6244 individually (Figure 6.6). Another tumour that presented intermediate response to treatment with AZD6244 alone, showed a drastic increase in apoptotic rates when treated with combinations of AZD6244 and aspirin. However, proliferation rates demonstrated only slight changes and therefore this tumour did not meet the criteria previously established for responding samples (Figure 6.7). Interestingly, I also found that one tumour that demonstrated a dose-dependent inhibition of proliferation and increase of apoptosis in response to treatment with AZD6244 alone, showed no effects on proliferation when treated with combinations of this agent and aspirin (Figure 6.8). This suggests that, in this particular tumour, aspirin reduced rather then increased sensitivity to AZD6244.

	Sample ID	Ki-67	Act. Casp.3	+ 100µM Aspirin Ki-67	+ 100µM Aspirin Act. Casp.3
114T	0uM AZD6244	1.0	1.0	1.0	1.0
	0.1uM AZD6244	0.8	2.0	0.00	0.7
	3uM AZD6244	1.4	1.0	0.47	2.7
115T	0uM AZD6244	1.0	1.0	1.0	1.0
	0.1uM AZD6244	1.0	1.6	0.55	3.6
	3uM AZD6244	0.0	0.2	0.36	2.8
116T	0uM AZD6244	1.0	1.0	1.0	1.0
	0.1uM AZD6244	0.4	1.6	0.73	0.8
	3uM AZD6244	0.6	0.9	0.58	0.8
117T	0uM AZD6244	1.0	1.0	1.0	1.0
	0.1uM AZD6244	0.3	0.6	0.29	2.4
	3uM AZD6244	1.5	1.0	1.14	2.6
118T	0uM AZD6244	1.0	1.0	1.0	1.0
	0.1uM AZD6244	0.7	0.6	0.75	0.8
	3uM AZD6244	0.4	1.6	0.71	1.5
120T	0uM AZD6244	1.0	1.0	1.0	1.0
	0.1uM AZD6244	0.5	1.3	0.36	0.1
	3uM AZD6244	0.0	0.2	0.03	0.3
122T	0uM AZD6244	1.0	1.0	1.0	1.0
	0.1uM AZD6244	0.1	0.2	0.07	0.3
	3uM AZD6244	1.7	1.2	0.19	0.2

Table 6.1: Aspirin increases sensitivity to AZD6244 in a subset of tumours. Table shows the effects of treatment with AZD6244 individually and in combination with aspirin on proliferation and apoptosis, obtained by immunohistochemical analysis. Colour scheme represents the changes observed in the levels of these markers: Green indicates a >2-fold decrease in proliferation or increase in apoptosis; Yellow indicates a 2-fold (+/-20%) variation in the levels of Ki-67 and act. caspase-3; and Red indicates that no changes or an increase in proliferation and decrease in apoptosis were observed in these tumours. Data shows that in a subset of tumours, aspirin increased sensitivity to AZD6244 resulting in a more dramatic decrease of proliferation and increase of apoptosis.

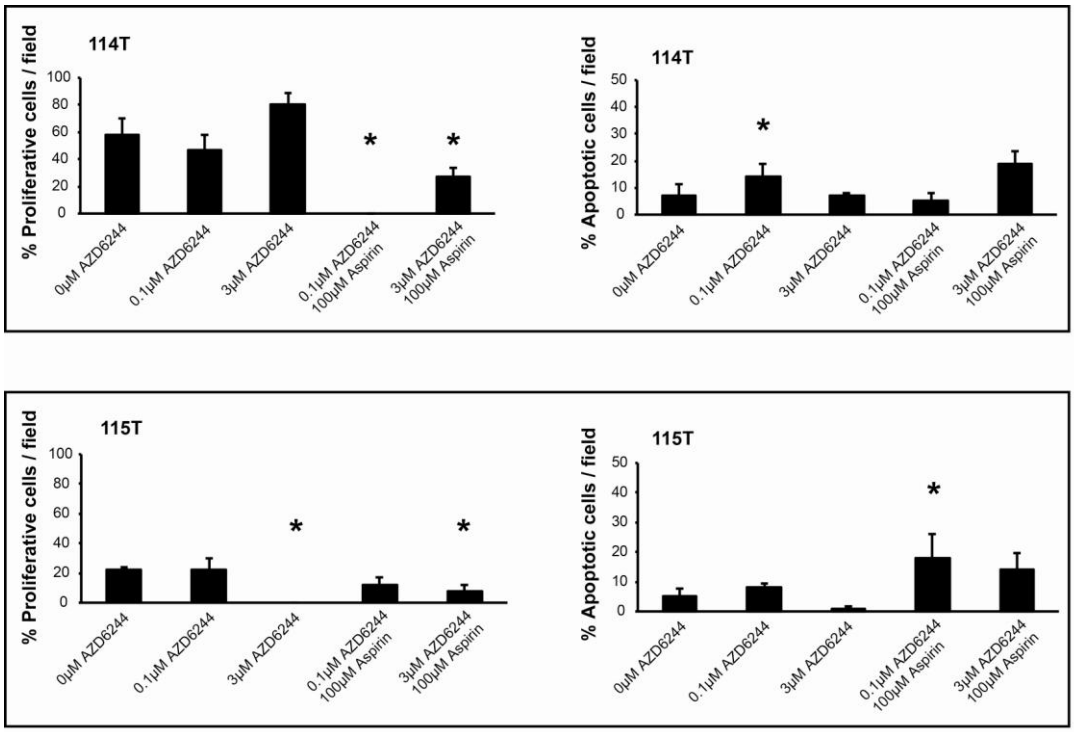


Figure 6.6: Aspirin increased sensitivity to AZD6244 in tumours displaying intermediate response to treatment with AZD6244 alone. Tumours were treated with AZD6244 alone and in combination with 100μM aspirin. Immunohistochemistry was performed to assess the effects on proliferation and apoptosis, as described in Figure 4.6. One tail Student's t test was used to calculate p values. Asterisk (*) indicates p≤0.05. Data shows that in these tumours, presence of aspirin increased sensitivity to AZD6244 translated by decreased proliferation and increased apoptosis when compared to effects observed with AZD6244 only.

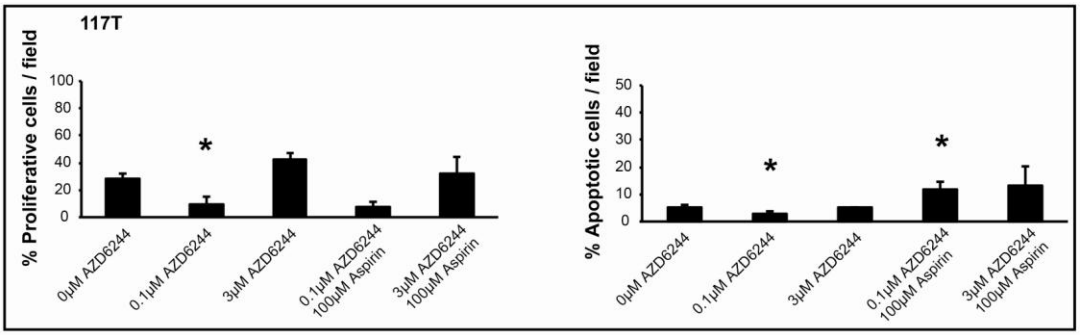


Figure 6.7: Combinations of AZD6244 and aspirin resulted in enhanced effect on apoptosis but not on proliferation. Tumour was treated with AZD6244 alone and in combination with 100μM aspirin. Immunohistochemistry was performed to assess the effects on proliferation and apoptosis, as described in Figure 4.6. One tail Student's t test was used to calculate p values. Asterisk (*) indicates p≤0.05. Data show that treatment with the combination regimes resulted in enhanced effect on apoptosis but not on proliferation. Addition of aspirin was not sufficient to fully increase sensitivity to AZD6244.

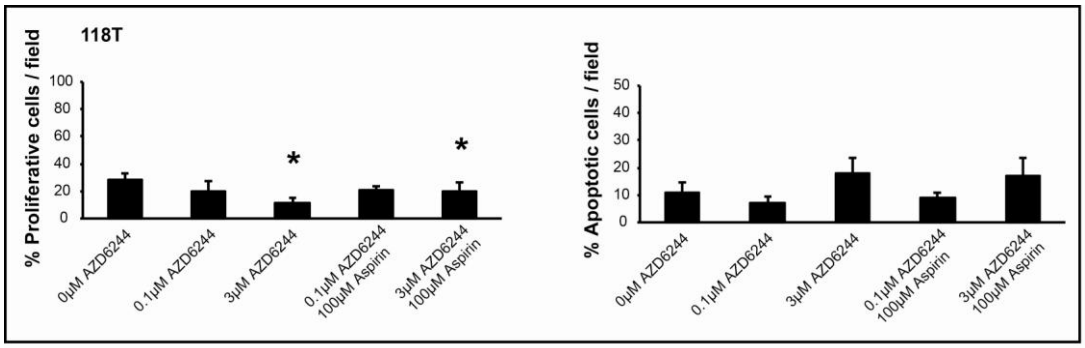


Figure 6.8: Treatment with combination regimes does not enhance response in tumours presenting increased sensitivity to AZD6244 alone. Tumour was treated with AZD6244 alone and in combination with 100μM aspirin. Immunohistochemistry was performed to assess the effects on proliferation and apoptosis, as described in Figure 4.6. One tail Student's t test was used to calculate p values. Asterisk (*) indicates p≤0.05. Data show that, in this particular tumour, the presence of aspirin reduced sensitivity to AZD6244.

I received one tumour that was large enough to treat in triplicate with AZD6244 alone and in combination with aspirin. I found that there was good consistency between the triplicate samples for the drug combination studies in that all three samples showed a dose-dependent inhibition of both proliferation and apoptosis with combinations of AZD6244 and aspirin (Figure 6.9). These data contrast to those obtained when the samples were treated with AZD6244 alone (see section 4.4). In samples treated with AZD6244 individually, expression of proliferation/apoptosis was not consistent between replicates. As mentioned in Chapter 4, this tumour was much larger in size (>10x) than all other tumours in the panel, which resulted in increased heterogeneity between the fragments cultured. These data suggest that different areas of the same tumour can present variable sensitivity / resistance to treatment, but also that variability might depend on the agent used. These data may suggest that while tumour response to AZD6244 alone may be quite variable, response to AZD6244 along with aspirin is more robust.

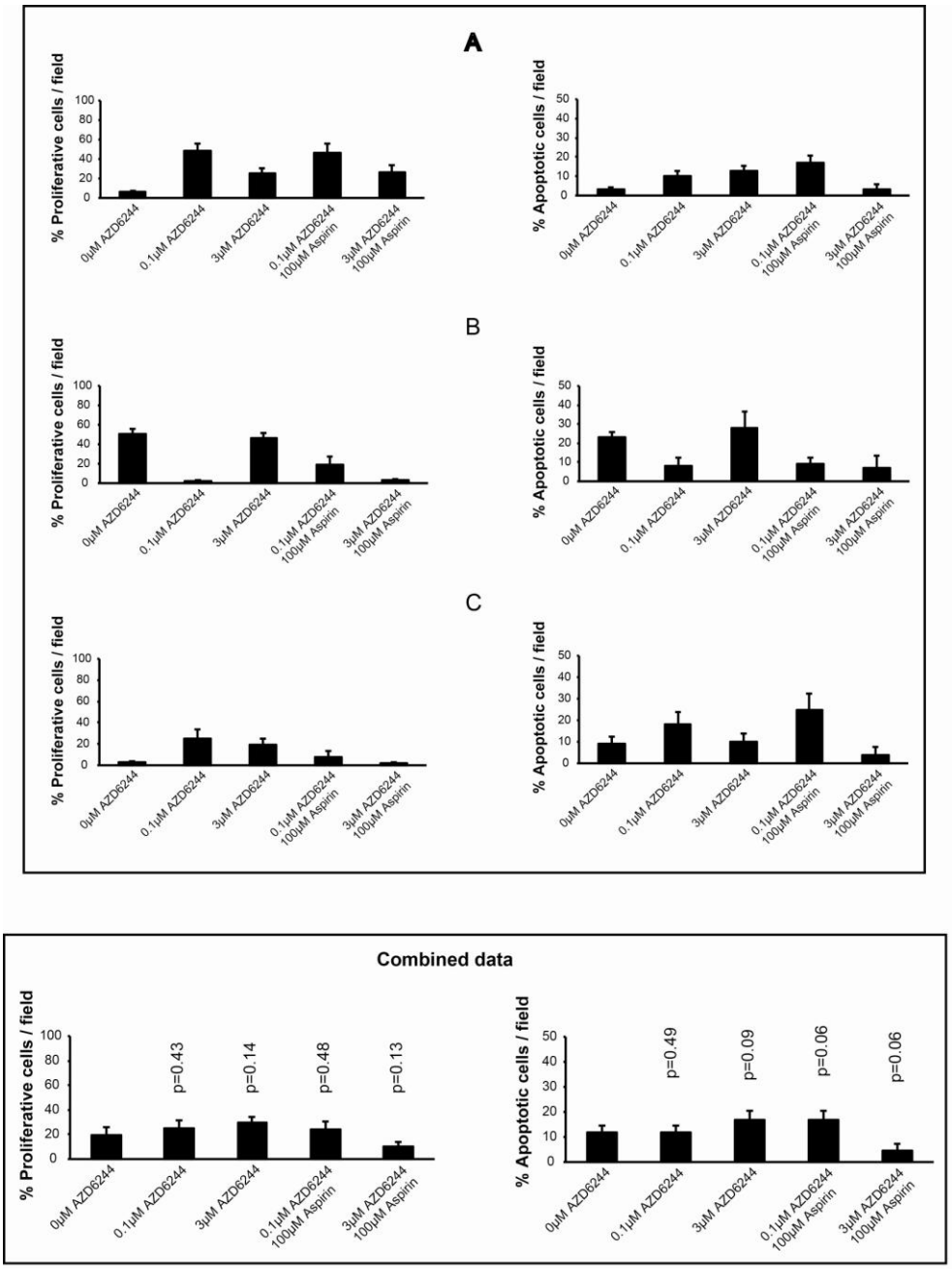


Figure 6.9: AZD6244 and aspirin effects on proliferation and apoptosis in a patient tumour treated in triplicate. A single tumour was divided into multiple fragments and treated with AZD6244 alone and in combination with aspirin, in triplicate. Immunohistochemistry was used to determine effects on proliferation and apoptosis. Procedure is described in Figure 4.6. One tail student's t test was used to calculate p values. Data shows that there is poor correlation between replicates treated with AZD6244 alone. However, good correlation between replicates is found in tumours treated with combinations of AZD6244 and aspirin, suggesting that response to these regimes is less variable between areas of the same tumour.

Discussion:

Data obtained with cell lines treated with combinations of AZD6244 and aspirin indicate that these drugs have synergistic effect, and this is more evident when low doses of both agents are used. However, greater synergism was observed in cell lines with intrinsic resistance to AZD6244 (RKO and HRT18) than in the more sensitive HCT116 cells. This suggests that different mechanisms are involved in the response to combination regimes and that these depend on individual characteristics of the cell population. Initial sensitivity to AZD6244 seems be an important factor in the response to combination therapy with this agent and aspirin. These data were very interesting and it prompted me to investigate the effect of AZD6244 and aspirin combinations in patient tumours.

Data obtained from patient tumours treated with combinations of AZD6244 and aspirin are in agreement with that obtained with colon cancer cell lines. Tumours presented distinct responses to the combination regimes, and these seemed to depend on the tumour's degree of sensitivity to AZD6244 alone. A small group of tumours showed increased sensitivity to treatment with combination therapy, suggesting that there is potential for this therapeutic option. However, data also indicates that combinations of AZD6244 and aspirin should not be used without further knowledge of the mechanisms involved in the response to combination regimes, and the individual characteristics behind differences observed between tumours.

One molecule that might be involved in the mechanism of response to combinations of AZD6244 and aspirin is Cyclin D1. Increased levels of this protein are thought to be related with resistance to AZD6244 (*114*). As aspirin causes Cyclin D1 degradation (*135*) sensitivity to AZD6244 might be increased by aspirin

in tumours presenting resistance to the MEK1/2 inhibitor alone. However, as previously mentioned, detection of this protein by western blot analysis proved challenging and therefore the levels of Cyclin D1 in tumours treated with combination regimes was not determined.

Blocking p-ERK1/2 activity does not impede upstream kinases like KRAS and BRAF from becoming active, and might even result in loss of the feedback mechanisms that control pathway homeostasis (see Section 1.4.1) which can result in continuous activation of these upstream members of the pathway. It is then possible that, in order to bypass pathway blockage downstream, KRAS and BRAF will activate other targets. These targets would be PI3K and IKK which can be activated by RAS and RAF proteins, respectively (*239, 245, 246*), promoting proliferation and survival, and the expression of anti-apoptotic genes. Targeting multiple pathways simultaneously would overcome such mechanisms. The presence of aspirin might counterbalance PI3K pathway activation by inhibiting PI3K, Akt or mTOR (*119-121*), and limit NFκB activity by promoting RelA translocation into the nucleolus (*134*). It would then be interesting to investigate if tumours that presented distinct responses to treatment with combinations of AZD6244 and aspirin, presented distinct patterns of activation of PI3K or downstream members of this pathway, or differential activation of IKK. It would also be interesting to investigate if nucleolar translocation of RelA occurred in all tumours treated with combinations of AZD6244 and aspirin, or only in those that showed increased sensitivity to the combination. Interestingly, the NFκB pathway has recently been implicated in the initiation of colorectal cancer through activation of RelA (*249*).

The presence of mutations in *KRAS* and *BRAF* could also be involved in the response to combinations of AZD6244 and aspirin, by promoting activation of IKK and the NFκB pathway. Unfortunately, in the panel of tumours treated with combinations regimes, only two samples carried *KRAS* mutations and neither responded to combination treatment. However, the sample size is too small to be able to draw any firm conclusions.

One tumour that I was able to treat in triplicate presented consistent results between replicates treated with combinations of AZD6244 and aspirin, but not with AZD6244 individually. This suggests that different parts of the same tumour might respond differently to treatment and that this might depend on the agent used. These data also suggests that while AZD6244 effects might be more variable, response to combination regimes is more robust.

It is important to stress that aspirin is commonly and widely used in the treatment and prevention of a variety of diseases. The data here presented, suggests that patients who take aspirin regularly may present altered response to other therapies in unanticipated ways.

Chapter 7: Discussion

The development of an *ex vivo* model of human colorectal tumours proved challenging and time consuming, and the long term growth of explants *ex vivo* presented significant limitations. Following these shortfalls, I proceeded to investigate the response of patient tumour explants to acute treatment with the MEK1/2 inhibitor AZD6244.

Using acute (short time point) treatments, I was able to consistently detect a reproducible pharmacodynamic response to AZD6244 in colorectal tumours from patients. This effect was evident by full inhibition of p-ERK1/2 within 1h of treatment with this agent, and it was observed in all tumours treated (see Figure 4.5). Furthermore, this effect was dose-dependent, and it was observed in two independent assays, western blot and RPPA analysis. Pharmacodynamic effects of treatment with aspirin, and combinations of this agent and AZD6244 were also observed using this method (see Figure 4.13 and 6.5). This suggests that an acute incubation can be useful to confirm the pharmacodynamic effects of drug treatment in patient tumour explants. Potentially, such a platform could also be used to examine the pharmacodynamic consequences of combination treatment strategies, helping to provide supporting evidence for their use in clinical development. It could, for example, be used to test the effects of anti-EGFR therapies in a wild type *KRAS* context, or effects of therapies that target the PI3K pathway, alone or in combination with AZD6244. However, it is less clear if the effects further down of pathway inhibition can be robustly measured using this study platform. This was investigated using RPPA analysis. However, I could not detect any significant changes in response to treatment with AZD6244 possibly due to the sensitivity of the assay or the choice of markers available.

When the phenotypic consequences of compound treatment were evaluated, much greater variability in response was observed. Consequently, strict classification criteria were required to stratify the tumours. I set stringent criteria so that tumours were considered sensitive to treatment only when reductions in cell proliferation and increases in apoptosis were observed in two separate fragments of the tumour and at both doses of AZD6244 tested. This minimised bias introduced by the experimental and analysis methods. Using these criteria, I was able to identify five tumours with apparently increased sensitivity to AZD6244. Analysis of the mutational status of *KRAS* and *BRAF* indicated that, at least in this model system, sensitivity to AZD6244 did not fully correlate with the presence of these mutations. However, the strict criteria used excluded many tumours, and when the responses induced by only low dose AZD6244 treatment (0.1μM) were examined, a better correlation with sensitivity and the presence of *KRAS* and *BRAF* mutations was observed. Whilst the availability of explant tissue hampered the number of samples that could be analysed, it was possible to treat one particularly large tumour in triplicate with AZD6244 and a combination of AZD6244 with aspirin. Whilst monotherapy treatment revealed intra-tumour variability, the effects observed in combination were more consistent. Further work on the assay is needed to examine the intra-tumoural variability in response to therapy in more detail. Tumours of larger size will be required for this, and these will be likely to be more heterogeneous. However, testing intra-tumoural variability in smaller samples remains challenging.

It has been suggested that AZD6244 effects can be enhanced by simultaneously targeting other pathways thought to be involved in the resistance to this agent. Finally, I tested response to treatment with combinations of AZD6244 and

aspirin, in cell lines and patient tumours, and found that these agents can have synergistic effect. However, sensitivity to combination regimes seemed to depend on sample sensitivity to AZD6244 alone.

Unfortunately, I could not develop an *ex vivo* model of human colorectal tumours that allowed for the growth of the explants and the study of response to longer-term treatment with AZD6244. However, the use of collagens gels showed some promise and I believe that it could have been improved by the use of tissue slices instead of fragments. The use of multicellular tumour spheroids obtained from disaggregated tumours also appeared to be a promising method despite the fact that it required extensive characterisation to determine if original tissue architecture was replicated in these structures, and optimisation of the analysis methods.

Regardless of the method used, growing tumours *ex vivo* is a technically difficult, time consuming and expensive process which might explain why these systems are not widely and routinely used. *Ex vivo* models are also limited by ethical issues and tissue availability; and with improvements in screening and detection of colorectal cancers the number and size of the tumours available is considerably reduced which limits the experimental options. Ideally, these platforms would be used together with clinical trials, so that responses observed in the lab could be correlated with those observed in the clinic, and validation of the method could be performed. However, this would require adaptation of the model system to the even smaller biopsy samples.

With the cancer stem cell field rapidly evolving, and recent reports that demonstrate the survival promoting role of stem cell marker Lgr5 in human

colorectal adenoma cell lines (*250*), it is also likely that colorectal cancer study models will soon take a different direction.

In summary, acute treatment of colorectal tumours can be used for pharmacodynamic readouts, but additional work is required to optimise for responsiveness, and robustly measure phenotypic effects. The ideal method would enable identification of markers of sensitivity and resistance early on in drug development, and contribute to the refinement of markers that could then be employed on diagnostic biopsies to stratify patients more widely in later trials. In other words, this model together with higher throughput analysis methods has the potential to contribute to the development of personalised medicine.

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